The signalling pathway of Bim_L and Bim_S, two isoforms of the BH3-only protein Bim, in apoptosis

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Erklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit selbständig und nur unter Zuhilfenahme der angegebenen Hilfsmittel angefertigt habe. Die praktischen Arbeiten wurden in der Abteilung Klinische und Molekulare Onkologie an der Robert-Rössle-Klinik, Charité, Campus Berlin-Buch der Arbeitsgruppe Prof. Dr. P. Daniel durchgeführt.

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Zusammenfassung

Ziel der vorliegenden Arbeit war es, die Rolle des pro-apoptotischen Proteins Bim am endoplasmatischen Retikulum (ER) und an den Mitochondrien zu untersuchen um den apoptotischen Signalweg dieses Mitgliedes der Bcl-2 Proteinfamilie näher aufzuklären.

Für diese Untersuchungen wurden zwei Isoformen von Bim verwendet, zum einen Bim_L, eine lange Version von Bim welches an den Motor Dynein Komplex gebunden ist, zum anderen die kürzere Form Bim_S, welches im Zytosol lokalisiert ist.

Um eine konditionale Expression von Bim zu erreichen, wurde Myc-markierte humane cDNA unter der Kontrolle des Tet-Off Systems in einen adenoviralen Vector kloniert.

Eine Überexpression von Bim_L und Bim_S induzierte in der Prostatakarzinomzelllinie DU145 unter Beteiligung der Proteine Bax und Bak apoptotischen Zelltod. Sowohl Bim_L, als auch Bim_S führte in stabil exprimierenden DU145-GFP-Bax und DU145-GFP-Bak Zellen zu einer Konformationsänderung und zu einem Zusammenclustern von Bax und von Bak. Eine Überexpression des anti-apoptotischen Proteins Bcl-2 in DU145 Zellen lokalisiert am endoplasmatischen Retikulum zeigte eine vollständige Hemmung der Bim-induzierten Apoptose, während Überexpression von Bcl-2 an den Mitochondrien nur eine partielle Hemmung herbeiführte. Bim Expression induzierte in DU-145-Bax und in DU145-Bak überexpremierenden Zellen den Zusammenbruch des mitochondrialen Membranpotentials. Dieses wurde ebenso in mit am ER lokalisiertem Bcl-2 in DU145 Zellen beobachtet. Bcl-2 lokalisiert an den Mitochondrien vermindert dagegen den Verlust des mitochondrialen Membranpotentials. Interessanterweise, war aber keines der beiden Bim Isoformen in der Lage Zelltod zu induzieren wenn Bcl-2 am ER exprimiert wurde, was die Wichtigkeit des endoplasmatischen Retikulums im Bim- vermittelten Apoptosesignalweg unterstreicht.

Um die durch Bim induzierten Prozesse am ER näher zu charakterisieren, wurde die Kalzium Freisetzung aus dem ER untersucht. Eine erhöhte zytosolische Kalzium Freisetzung konnte durch Bim_S Überexpression induziert werden und ging zeitlich einem Zusammenbruch des mitochondrialen Membranpotentials voraus. Proteinanalysen mittels Western Blot zeigten eine Hochregulierung von ER-Stress Proteinen nach Bim Überexpression. Die Bim-induzierte DNA-Fragmentierung wurde von einer Cytochrom c Freisetzung aus den Mitochondrien und der Prozessierung und Aktivierung von Caspase-9, -3 und -8 begleitet. Die Spaltprodukte dieser Caspasen wurden einerseits im Western Blot, andererseits durch fluoreszenzmarkierte Peptide, die an die Spaltform der erwähnten Caspasen binden, bestimmt. Mit einem Breitband-Caspase Hemmer konnte der Bim-induzierte Zelltod vollständig gehemmt werden, was zeigt, dass Caspasen für die

Exekution der Apoptose essentiell sind.

Durch Verwendung eines spezifischen Hemmers der Initiator Caspase-8 konnte der Zusammenbruch des mitochondrialen Membranpotentials nach Bim_S Induktion gehemmt werden, was auf eine wichtige Rolle der Caspase-8 oberhalb des Mitochondriums hindeutet.

Zusammenfassend kann gesagt werden, dass Bim, parallel zum mitochondrialen Signalweg, ER-Stress auslöst. Die Ergebnisse lassen darauf schliessen, dass Bim eine effektive Apoptose durch die Interaktion des endoplasmatischen Retikulums der Aktivierung von Caspasen und der Mitochondrien induziert.

Abbreviations

A. bidest	Aqua bidestillata
AIF	apoptosis inducing factor
ANT	Adenine Nucleotide transporter
Apaf-1	apoptosis associated factor1
APS	Ammoniumperoxodisulfate
Bak	Bcl-2 homologous antagonist killer
Bap31	Bcl-2-associated protein-31
Bax	Bcl-2 associated protein X
BCA	Bicinchonin acid
Bcl-2	B-cell lymphoma / leukemia-2 gene
BGHpolyA	Bovine growth hormone polyadenylation signal
BH	Bcl-2 homology
Bik/Nbk	Bcl-2 interacting killer/Natural born killer
BSA	Bovines serum Albumin
CAPS	3-Cyclohexylamino-1-propansulfon acid
CARD	caspase recruitment domain
CMV	Cytomegalovirus
ColE1 ori	High-copy number origin of replication for *E. coli*
C-Terminus	carboxy-terminus
CVL	crude virus lysate
Cyt *c*	cytochrome *c*
Da	Dalton
DEAE	Diethylaminoethanol
DD	death domain
DED	death effector domain
Diablo	Direct IAP binding protein with low pI
DISC	death inducing signalling complex
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid

dNTP	2´-Desoxynukleosidtriphosphat
$\Delta\Psi m$	mitochondrial membrane potential
DTT	Dithiothreitol
E. coli	Escherichia coli
ECL	Enhanced chemoluminescence
EDTA	Ethylendiamine-tetraactylacid
ER	endoplasmic reticulum
EtBr	Ethidiumbromide
EtOH	ethanol
FBS	fetal bovine serum
FITC	Fluorescein-isothiocyanat
fmk	Fluoromethylketon
FSC	Forward scatter
h	hour
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]-Ethansulfonic acid
HRP	horse radish peroxidase
HtrA2	High temperature requirement A2
IAP	inhibitor of apoptosis protein
IRES	Internal Ribosome Entry Site
JC-1	5,5‘,6,6‘tetrachloro1,1‘,3,3‘tetraethylbenzimidazolyl carbocyaniniodide
LB-medium	Luria-Browth-Medium
MEF	Murine embryonic fibroblasts
min	minute
MOI	multiplicity of infection
MTS	Mitochondrial targeting sequence
N-Terminus	Amino-terminus
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly(ADP-ribose) Polymerase
PBS	Phosphate buffered saline
PCMV	full length “immediate early” promotor of cytomegalovirus
PCR	Polymerase chain reaction

Pfu	Plaque forming unit
PI	propidium iodide
PIDD	p53-induced protein with Death Domain
PminiCMV	minimal “immediate early” promotor of cytomegalovirus
PMSF	Phenylmethylsulfonylfluoride
PTP	permeability transition pore
RAIDD	RIP-associated ICH-1/CED-3-homologous protein with Death Domain
RING-domain	Really Interesting New Gene
Rpm	rounds per minute
RT	room temperature
s	Second
SDS	Sodium dodecylsulfate
SERCA	Sarco(Endo)plasmatic reticulum Ca^{2+}-ATPase
Smac	second mitochondria activator of caspases
SSC	Side scatter
TAE-buffer	Tris-Acetat-EDTA-buffer
TEMED	N, N, N‘, N‘-Tetramethylethylendiamin
TM	Transmembrane domain
TNF	tumour necrosis factor
TRE	Tetracycline-responsive element
Tris	Tris-(hydroxymethyl)-aminomethan
tTA	Tetracycline-controlled transactivator
U	Unit
UPR	unfolded protein response
V	Volt
v/v	volume / volume
VDAC	voltage dependent anion channel
Vol.	Volume
w/v	weight / volume
XIAP	X-chromosome-linked Inhibitor of Apoptosis Protein
X-Gal	5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranosid

1 Summary

The aim of this thesis was to investigate the role of the pro-apoptotic BH3-only protein Bim, at the endoplasmic reticulum (ER) and the mitochondria and additionally to reveal the apoptotic signalling pathway of this Bcl-2 family member.

For this purpose, a full length human myc-tagged Bim cDNA was cloned into an adenoviral vector, which allows for the conditional expression of the transgene under the control of a Tet-Off-system. Two different kinds of Bim isoforms were used for these investigations. One was Bim_L, the long version of the Bim protein, which is bound to the motor dynein complex of the microtubule and the other one was Bim_S, the short splicing variant, which is localized in the cytosol.

The enforced expression of each of these two isoforms in the prostate cancer cell line DU145, showed the capability of Bim_L and Bim_S to induce apoptosis via either Bak or Bax. Further, expression of both Bim isoforms in DU145 cells, stably expressing GFP-Bax or GFP-Bak, confirmed that Bim induces redistribution and clustering of Bax and Bak. Also, both DU145-Bax and DU145-Bak cells showed breakdown of the mitochondrial membrane potential upon overexpression of either Bim isoforms. This effect was also observed in DU145 cells overexpression the anti-apoptotic protein Bcl-2 at the ER. However, targeting Bcl-2 to the mitochondria gave these cells partial resistance to Bim-induced mitochondrial permeabilization. These findings indicated the employment of the intrinsic apoptotic pathway for cell death induction upon Bim signalling. Nevertheless, expression of Bcl-2 at the mitochondria partially suppressed Bim-induced apoptosis whereas Bcl-2 was targeted to the ER entirely prevented cell death induction by Bim underlining the importance of the ER in the Bim-mediated cell death pathway. To examine the events at the ER in the Bim pathway, the calcium effluxes from the endoplasmic reticulum into the cytosol were assessed. Increased cytosolic calcium levels could be detected upon Bim_S expression and preceded activation of the mitochondria. Further, Western blot analysis showed an upregulation of ER stress proteins upon Bim expression. Bim-induced DNA-fragmentation was accompanied by cytochrome *c* release form the mitochondria and the processing and activation of caspase-9, -3 and -8. Cleavage products of these caspases were detected by Western blot analysis and their activation were shown by binding of FITC-labelled substrates against the indicated caspases. In addition, the complete inhibition of Bim-induced cell death by a pan caspase inhibitor revealed that caspases are crucial for the execution of apoptosis. Inhibition of caspase-8 by a specific inhibitor did not lead to the breakdown of the mitochondrial membrane potential upon Bim_S overexpression suggesting that capase-8 takes an important place upstream of the mitochondria.

In conclusion, Bim induces the mitochondrial apoptotic pathway and, in parallel, triggers ER stress. It seems that Bim mediates cell death through the interaction of the mitochondria, the ER and caspase activation. Induction of a secondary mitochondrial activation by an ER-mitochondria cross-talk leads to the amplification of the apoptotic death signal.

2 Introduction

2.1 Cell death

2.1.1 Necrosis

Cell death occurring passively, in an unregulated and uncontrolled fashion is called necrosis (figure 1). This process is initiated when cell damage is too severe to overcome and when the cell is not able to activate the energy-dependent apoptotic pathway. Necrosis is marked by cell and organelle swelling due to augmentation of the membrane permeability. This leads to the release of lysosomes into the extra cellular matrix (Kerr, et al., 1994; Wyllie, et al., 1980). Intracellular contents are thrown into the extra cellular matrix often causing inflammation in the neighbouring cells.

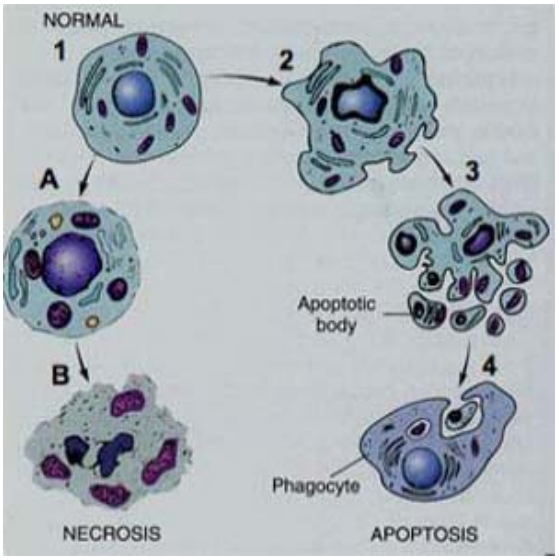

Figure 1: morphological changes during apoptosis and necrosis

1) Normal cell
2) Shrinking of the cell, condensation of the chromatin and membrane blebbing
3) Nuclear fragmentation and formation of apoptotic bodies
4) Phagocytosis of the apoptotic bodies
A) Swelling of the organelles
B Disruption of the cell membrane
(Kerr, et al., 1994)

2.1.2 Apoptosis

The term apoptosis was established by Kerr (Kerr, et al., 1972). Apoptosis is a selective form of cell death that consists of morphological and biochemical changes. It is a highly conserved process, from C. elegans to man. It describes a controlled process consisting of shrinkage of the cell, blebbing of the plasma membrane, condensation of the chromatin

leading to DNA fragmentation and eventually formation of so-called apoptotic bodies (Wyllie, et al., 1984) (figure 1). These are eliminated by phagocytosis to avoid inflammation (Fadok, et al., 2001). Apoptosis allows elimination of unwanted, aged, misplaced or damaged cells. Therefore, apoptosis is a crucial process in embryonic development, tissue homeostasis, differentiation, proliferation and immune response (Vaux and Korsmeyer, 1999). Developmental cell death occurs in a programmed fashion and is referred to as “programmed cell death” (type I cell death). However, this process can also be induced by exogenic factors like DNA-damaging agents, UV-irradiation and nutrition depletion. Inhibition or abnormal regulation of apoptosis underlies many disorders such as cancer and autoimmunity (Bakhshi, et al., 1985).

2.1.3 Autophagy

In cell death type II or autophagy, cell death occurs by degradation, where the cytoplasm or damaged organelles are recycled (Shintani and Klionsky, 2004). Autophagy is defined by the formation of the autophagosome, a double- or multi-membrane bound vacuole (Dunn, 1990). Fusion with lysosomes results in autophagolysosomes degrading their contents. Apart from this function, autophagy is engaged in cellular remodelling during differentiation and metamorphosis as well as aging, muscular disorder and neurodegeneration (Shintani and Klionsky, 2004). Autophagy might be an attempt to protect the cells against mitochondrial permeability transition during oxidative stress or mitochondrial calcium overkill (Rodriguez-Enriquez, et al., 2004).

2.2 Apoptotic pathways

Three main starting points of apoptosis are known, death receptors, mitochondria and the endoplasmic reticulum (Nakagawa and Yuan, 2000). These pathways are largely independent but can establish crosstalk since there are some junctions along their pathways.

2.2.1 The death receptor pathways

Extra cellular receptors transmit external signals into the cell. Besides signals that lead to proliferation and differentiation, cytotoxic signals are taken up by these receptors e.g. to eliminate target cells or excess immune cells after an immune response (Ashkenazi and Dixit, 1998; Schulze-Osthoff, et al., 1994). Initiation of the extrinsic or death receptor pathway (figure 2) occurs upon binding of extra cellular death ligands to the tumour

necrosis factor (TNF) super-family of plasma membrane death receptors. Members of this receptor family are Fas/CD95, TNFR1, TRAIL receptors DR3, DR4 and DR5. They possess a cysteine rich extra cellular domain (Smith, et al., 1994). Upon binding of the death ligand, the death receptors form trimers. The intracellular death domain (DD) of the receptor recruits adaptor proteins like FADD (Fas Associated Death Domain) or TRADD (TNF-receptor associated protein with death domain) (Chinnaiyan, et al., 1995). In turn, these adapter proteins bind, with their N-terminal death effector domain (DED), to procaspase-8 leading to formation of a complex called death inducing signalling complex (DISC) (Muzio, et al., 1996). In this complex, monomeric caspase-8 becomes at least a dimer leading to autoprocessing and activation. Activated caspase-8 can proteolytically process and activate the effector caspase-3, -6, and -7 to amplify the death signal. Furthermore, caspase-8 can cleave Bid, a pro-apoptotic protein, which can initiate the mitochondrial apoptotic pathway (Li, et al., 1998; Luo, et al., 1998). Regulation is mediated by the caspase-8 inhibitory proteins FLIP (flice inhibitory protein) that exists in two splicing variants, the long form $FLIP_L$ and the short one $FLIP_S$. They contain two DEDs, but they do not have proteolytic activity (Thome, et al., 1997; Thome and Tschopp, 2001). Both cellular isoforms possess significant anti-apoptotic activity once they are part of the DISC. Overexpressed FLIP can block autoproteolytic activation of procaspase-8 by binding competitively to FADD (Thome and Tschopp, 2001).

2.2.2 The mitochondrial death pathway

The intrinsic pathway is regulated by proteins of the Bcl-2 family and is therefore referred to as the mitochondrial pathway, because the mitochondria play a central role (figure 2). Several stimuli can lead to activation of the mitochondrial cell death pathway such as cytotoxic drugs, heat shock, ionising, DNA damage and growth factor withdrawal. These stimuli trigger Bax and Bak activation, which subsequently mediate the permeabilization of the outer mitochondrial membrane and release of distinct proteins. Among them are cytochrome *c*, Smac/Diablo, apoptosis inducing factor (AIF), endonuclease G and Omi/HtrA2 (van Loo, et al., 2002). Released cytochrome *c* mediates the formation of a protein complex called apoptosome, which consists of Apaf-1 (apoptosis protease activating factor 1), initiator caspase-9 and (d)ATP (Li, et al., 1997; Liu, et al., 1996; Saleh, et al., 1999). Binding of Apaf-1 to cytochrome *c* increases the affinity of Apaf-1 to ATP, which is providing the energy for apoptosome formation (Hu, et al., 1999). During apoptosome formation procaspase-9 is activated and, in turn, causes the activation of the effector caspase-3 and caspase-7 (Rodriguez and Lazebnik, 1999). Further, cytochrome *c* release is accompanied by a mitochondrial permeability shift, acidification of the cytosol

and ion fluxes (Daniel, et al., 2003). Released endonuclease G translocates to the nucleus, where it digests DNA caspase-independently (van Gurp, et al., 2003). Moreover, activation of released Smac/Diablo results in dimerization. Smac/Diablo contributes to caspase activation by restraining inhibitor of apoptosis proteins (IAPs). As implied by the name, these proteins inhibit activation of pro-caspases. In his task Smac/Diablo is supported by Omi/HtrA2 (van Gurp, et al., 2003). The death receptor signalling pathway is connected to the mitochondrial cell death pathway by the BH3-only protein Bid when cleaved by caspase-8 (Gross, et al., 1999). This truncated Bid may bind to Bax or Bak thereby inducing a conformational change in these proteins and the insertion of them into the outer mitochondrial membrane. It also sequesters anti-apoptotic Bcl-2 family proteins. This is followed by the permeabilization of the mitochondrial membrane and consequently the amplification of the mitochondrial pathway. Despite of this connection the pathways function mostly independently form each other (Bouchon, et al., 2000). The anti-apoptotic proteins of the Bcl-2 family can block the intrinsic pathway. These proteins can prevent cytochrome *c* release and caspase activation (Kluck, et al., 1997).

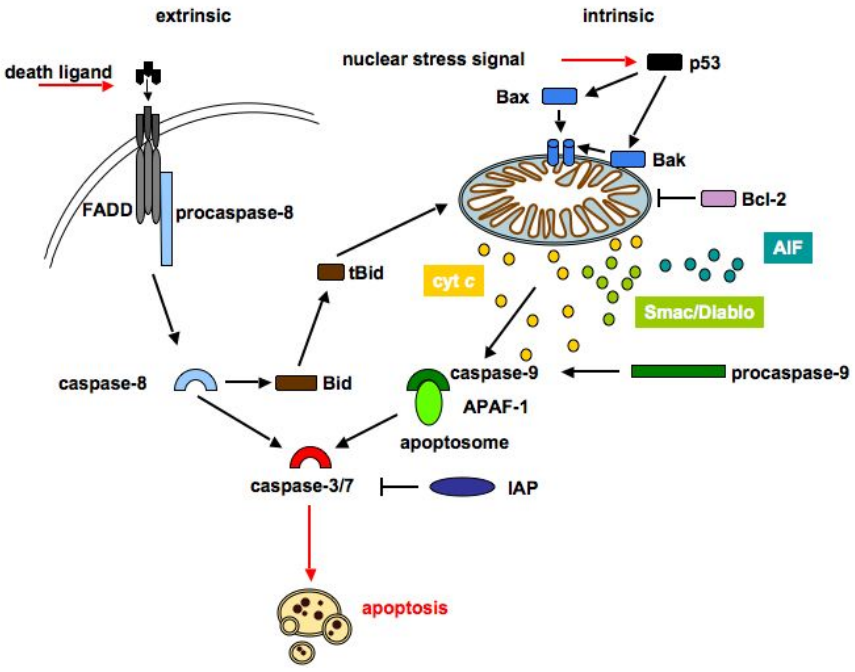

Figure 2: The mitochondrial and death receptor apoptotic pathway

*The mitochondrial (**intrinsic**) apoptotic signalling pathway is initiated by cell damaging events, upon which pro-apoptotic members of the Bcl-2 family are activated and translocate to the mitochondria to neutralize anti-apoptotic proteins. Permeabilization of the mitochondria causes the release of cytochrome c. Released cytochrome c associates with Apaf-1 and procaspase-9 in the presence of dATP to form the apoptosome. Activated caspase-9 triggers a caspase cascade leading to apoptosis.*
*The death receptor (**extrinsic**) pathway is activated when ligands of the TNF family bind to their receptors on the cell surface. Binding of the ligand induces trimerization of the receptor and recruitment of the adaptor protein FADD and caspase-8. Within this complex, caspase-8 is activated and in turn cleaves and activates casapse-3. The two pathways are mostly independent, but in type II cells the two pathways can be linked via cleavage of Bid by caspase-8, caspases-3 or -10. Truncated Bid activates the mitochondrial apoptotic pathway.*

2.2.3 The endoplasmic reticulum pathway

The endoplasmic reticulum is responsible for the maintenance of the calcium homeostasis and is also the major intracellular calcium storage. The uptake of Ca^{2+} into the lumen of the ER is managed by energy-dependent SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase). The release is handled by IP_3 (Inositol 1,4,5-Triphosphat (IP_3)-regulated receptors or ryanodin (RyR) Ca^{2+} receptors (Berridge, et al., 2000). Furthermore, the ER is the main compartment for protein synthesis, folding, targeting and trafficking. The ER contains numerous chaperone proteins, a high level of calcium and an oxidative environment to carry out these functions efficiently (Rao, et al., 2004). Changes in Ca^{2+} levels or accumulation and aggregation of un- or misfolded proteins lead to ER stress, which is gauged by the ER stress sensors IRE1, PERK and ATF6. To restore normal ER function, the unfolded protein response (UPR) is initiated (Szegezdi, et al., 2006). Excessive ER stress forces the unfolded protein response to activate diverse pathways that eventually lead to apoptosis (Ferri and Kroemer, 2001). Also, prolonged ER stress is involved in the pathogenesis of some neurodegenerative disorders that feature misfolded proteins (Rao, et al., 2002). ER stress can also be elicited by several agents including tunicamycin a specific N-glycosylation inhibitor, Brefeldin A, an inhibitor of the protein transport from ER to Golgi and thapsigargin, which blocks Ca^{2+} uptake by inhibiting the SERCA (Lee and East, 2001). The answer to these stresses is the upregulation of ER chaperons, including the glucose regulated protein GRP78 also referred to as BiP (immunoglobulin heavy chain-binding protein) and the transcription factor CHOP (C/EBP homologous protein). They have both anti-apoptotic features and regulate the ER stress sensors (Lee, 2005). To relief the ER stress, GRP78/BiP accelerates protein folding in the ER lumen (Momoi, 2004). CHOP sensitizes cells to ER stress by downregulation of Bcl-2 and activation of GADD34 (protein phosphatase 1 (PP1)-interacting protein) and ERO1alpha, an ER oxidase (Li, et al., 2006). In summary, these proteins facilitate protein folding and prevent aggregation.

In mice, chaperones present the signal to capase-12, which is on the cytoplasmic site of the ER membrane (Szegezdi, et al., 2003). The role of caspase-12 in human is not known since it is expressed in a truncated form and is not functional (Nakagawa, et al., 2000). Caspase-4 was proposed to fulfil this function in human, but it is still under debate. It was shown to be localized at the ER and be activated by ER stress. Its mechanism though, is still not fully understood (Hitomi, et al., 2004). Capase-12 is activated upon ER stress, but neither upon death receptor nor mitochondrial apoptotic signals (Nakagawa, et al., 2000). Apart from the discussion about activation of murine caspases-12 upon ER stress, there is also the possible involvement of caspases-3 being involved in Ca^{2+} homoeostasis. Caspase-3 cleaves IP3 receptors in Jurkat cells and thus decreases the activity of the IP3

receptor under apoptotic circumstances (Hirota, et al., 1999).

2.3 Calcium mobilization

Calcium from the ER is not just an ER stress signal it could also be involved in the regulation of Ca^{2+} influx, ER protein folding and chaperone interaction, gene expression and regulation of nuclear pore opening. Calcium elevations may be mediated by ER proteins, BAP31 (Breckenridge, et al., 2003), or tBid, which enhance transmission of IP_3 mediated Ca^{2+} signals to mitochondria (Csordas, et al., 2002). Ca^{2+} released from the ER is taken up by the mitochondria and accumulates in the matrix and this may reset in cytochrome *c* release. Ca^{2+} traverses the outer mitochondrial membrane primarily through the voltage dependent anion channel (VDAC). Ca^{2+} interacts with cyclophilin D, a component of the permeability transition pores, to induce their opening of the permeability transion pore (Basso, et al., 2005). Calcium overload of the mitochondria has been linked to the recruitment of Drp1, that has been implicated in mitochondrial fragmentation (Breckenridge, et al., 2003; Rudner, et al., 2002). Consequences of the pore opening are the loss of membrane potential and re-release of Ca^{2+}. When the elevated cytoplasmic Ca^{2+} level persists, the permeability transion pore stays open and allows accumulation of solutes in the mitochondrial matrix. The entry of solutes leads to the extension of the mitochondrial matrix and to rupture of the outer mitochondrial membrane, thereby to releasing the intermembrane space content (Green and Kroemer, 2004). Changes in Ca^{2+} levels appear to be regulated by both pro-and anti-apoptotic members of the Bcl-2 family. Bax, Bak and Bcl-2 are localized at the outer mitochondrial as well as at the ER membrane. At both membranes they regulate fluxes of calcium, meaning the release from the ER and the subsequent uptake by the mitochondria, while Bax and Bak support calcium release and Bcl-2 and Bcl-x_L antagonize it (Garrido, et al., 2006). Bcl-2 was shown, however, to diminish calcium levels in the ER and to stimulate accelerated re-uptake into the ER or into the mitochondria. In this way, the Ca^{2+} concentration does not cross an intracellular threshold after an apoptotic insult. It might be that blockade of Bcl-2 and Bcl-x_L interaction with IP_3 (inositol 1,4,5-trisphosphate) receptors helps the effect of Bax and Bak (Chen, et al., 2004; White, et al., 2005). IP_3 receptors are found on the ER membrane, where they regulate the mobilization of Ca^{2+} stores (Berridge, 2005). Recent studies point to a link between the function of cytochrome *c* and IP_3 triggered Ca^{2+} mobilization (Boehning, et al., 2005). Overexpression of Bcl-2 partially prevents rise of cytoplasmic Ca^{2+} levels upon exposure to exogenous ceramide, staurosporin, thapsigargin or growth factor depletion (Distelhorst and McCormick, 1996). Moreover, Bcl-2 targeted to the ER (Zhu, et al., 1996) averted apoptosis stimulated by ceramide, irradiation, thapsigargin and the upregulation of Bax and Bad (Annis, et al., 2001; Rudner,

et al., 2001; Thomenius, et al., 2003; Wang and Spector, 2001). A model for calcium release from the ER is given in figure 3.

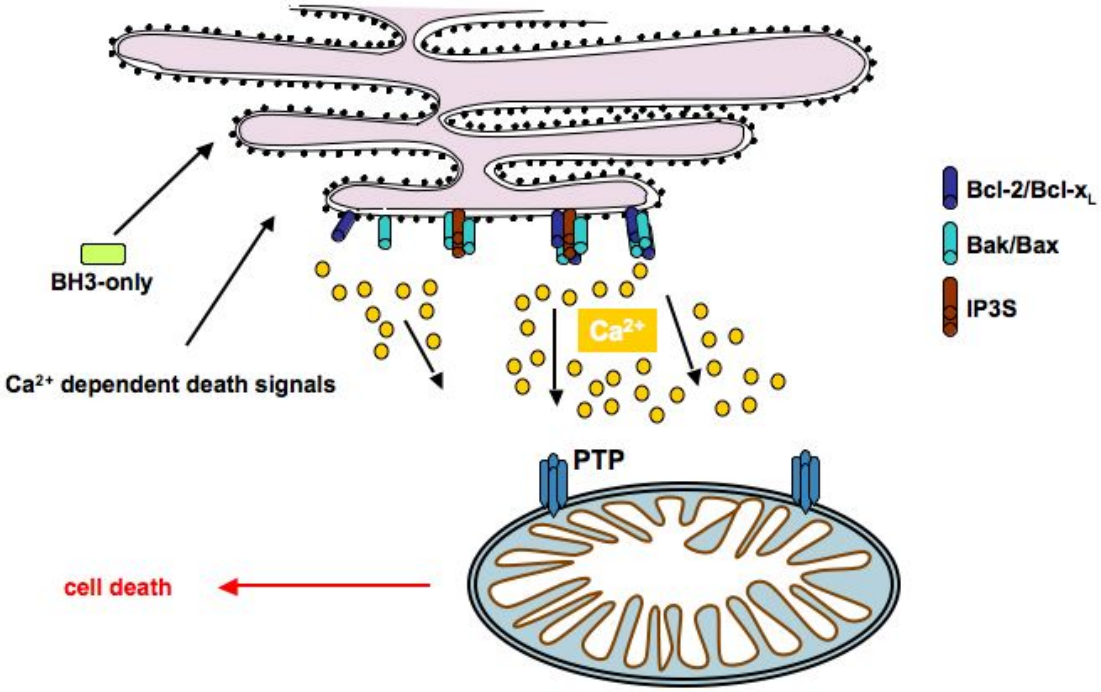

Figure 3: ER stress and Calcium release

Apoptotic signals targeting the ER may induce Ca^{2+} release to regulate mitochondrial activation by opening its permeability transition pore (PTP).

2.4 Activation of the mitochondria

Mitochondria produce ATP, maintain the equilibrium in-between ions and regulate apoptosis. As already mentioned (see 1.2.2), upon apoptotic signals mitochondrial proteins are release form the intermembrane space into the cytosol. Among these proteins are cytochrome *c* (Liu, et al., 1996), Smac/Diablo (Adrain, et al., 2001), Omi/HtrA2 (Suzuki, et al., 2001), AIF (Cande, et al., 2002) and endonuclease G (Li, et al., 2001). In order to release these proteins the outer mitochondrial membrane needs to be permeabilized.

2.4.1 Mitochondrial outer membrane permeabilization

The mechanisms of mitochondrial outer membrane permeabilization have not yet been illuminated in all details. This leaves room for several models. The two most popular ones are the permeabilization of the inner membrane by so far enigmatic mechanisms, and the insertion of the multi-domain Bcl-2 proteins Bax and Bak into the outer mitochondrial

membrane.

Permeabilization of the inner mitochondrial membrane is achieved by formation of a channel spanning through both membranes of the mitochondria (Crompton, 2000). Each of these channels, so-called permeability transition pores, has been suggested to consists of the voltage-dependent anion channel (VDAC), an outer membrane protein, the adenine nucleotide translocator (ANT), in the inner membrane, and cyclophilin D in the matrix (Green and Kroemer, 2004). Increased cytosolic Ca^{2+} or reactive oxygen species promote the opening of these more or less functionally defined permeability transition pores. Once the pore is open it allows the osmotic driven influx of water, ions and other small molecules into the matrix. These activities leading to swelling of the mitochondria, and are termed mitochondrial permeability transition (Zamzami and Kroemer, 2001). Now, rupture of the outer mitochondrial membrane is induced and eventually the outer membrane is permeabilized, spilling the mitochondrial content into the cytosol (Armstrong, 2006). This goes along with the loss of the mitochondrial membrane potential. VDAC, the core of the permeability transition pore, is regulated by Bax, Bak and Bcl-2 (Chandra, et al., 2005). In another model, pro-apoptotic Bax and Bak form pores in the outer mitochondrial membrane by oligomerization. But first, BH3-only proteins induce an allosteric conformational change in Bax and Bak. Conformational change exposes the N-terminal region of Bax and is needed for integration into the membrane (Korsmeyer, et al., 2000). Now, Bax is able to translocate to the mitochondria and stably insert into the membrane. They form large multimeric pores in the outer mitochondrial membrane. Intermembrane space proteins can escape through these pores into the cytosol. In this model, the inner membrane and the matrix would not be affected and remain intact. The crucial role of Bax and Bak in mitochondrial membrane permeabilization becomes evident by the fact that double knockout cells do not undergo outer membrane permeabilization in response to apoptotic stimuli (Armstrong, 2006). The pore forming model embarked, when structural similarities of pore forming bacterial toxins and multi-domain proteins were found (Muchmore, et al., 1996). But not only Bax and Bak are included in this model. Truncated Bid is also believed to form a pore into the outer mitochondrial membrane by oligomerization. Therefore it might function like Bax and Bak regarding permeabilization of the mitochondria (Henry-Mowatt, et al., 2004). While Bax and Bak induce the release of apoptotic factors from the mitochondria, Bcl-2 and Bcl-x_L block outer membrane permeabilization and thereby avoid the release of these factors (Daniel, et al., 2003). Due to interactions between pro- and anti-apoptotic proteins, the binding affinity and the stochiometry in-between them is a crucial factor in induction of apoptosis.

2.4.1.1 Release of cytochrome c

It is not exactly known how cytochrome *c* is translocated through the mitochondrial outer membrane, but there is evidence that the Bcl-2 family members are the main regulators of cytochrome *c* release. Bcl-x_L, Bax and Bid share similarities in their transmembrane domains, which they have in common with bacterial colicin toxins and diphtheria toxins. This led to the assumption that Bcl-2 proteins are also able to form pores. On one hand, there is the model already described above, implying that Bax forms pores upon oligomerization and integration into the outer mitochondrial membrane. On the other hand it has been suggesting that Bax and Bak interact with VDAC and thereby stimulates the opening of this channel, although the mechanism is not known (Marzo, et al., 1998; Saito, et al., 2000). This model is supported by the finding that inactive Bak is associated to VDAC, but can be freed by anti-apoptotic proteins that bind to VDAC2 (Chandra, et al., 2005). Other reports doubt the pore formation of Bax and Bak, although confirming their ability to oligomerize. Additionally to these two models, it was shown that the fragmentation of the mitochondrial network during apoptosis is an essential event for mitochondrial breakdown. Moreover, it was proposed that Bax plays a critical role in mitochondrial fragmentation (Karbowski, et al., 2002). Also, it was shown that the truncated form of Bid can release apoptotic factors from the mitochondria by forming pores at the outer membrane, when the extrinsic apoptotic pathway is activated (Zamzami, et al., 2000). Further, it was reported that the pro-apoptotic activity of BH3-only proteins induce cytochrome *c* release without permeability transition (Shimizu and Tsujimoto, 2000).

There are arguments for and against the models mentioned. Most likely, different apoptotic stimuli induce distinct mechanisms for permeabilization of the outer mitochondrial membrane.

2.4.1.2 Release of pro-apoptotic factors

Not only cytochrome *c* is set free upon disruption of the outer mitochondrial membrane. Also other apoptosis-inducing factors are liberated from the mitochondria.

AIF (apoptosis inducing factor) is a flavoprotein, which translocates to the nucleus and is participating in chromatin condensation and DNA-fragmentation in a caspase-dependent manner (Daugas, et al., 2000; Lorenzo, et al., 1999; Susin, et al., 1999). Its redox activity is not important for its apoptotic effect (Miramar, et al., 2001). These data are, however, discussed in a highly controversial fashion.

Endonuclease G is a DNA degrading enzyme important for DNA-repair in mitochondria. It

is released from the matrix and migrates to the nucleus and degrades DNA in a caspase independent way (van Loo, et al., 2002).

Omi/HtrA2 (high temperature requirement A2) is a serine protease, which acts as chaperone for correction of misfolded proteins or their degradation, but also inhibits IAPs (inhibitor of apoptosis proteins). Its mitochondrial signal sequence targets it to the intermembrane space, where this targeting-signal is being cleaved off and, the remaining protein is transformed into a mature protease. In the cytosol, Omi/HtrA2 facilitates caspase-dependent apoptosis, catalytic cleavage of IAPs, and permeabilization of the outer mitochondrial membrane (Faccio, et al., 2000; Hegde, et al., 2002; Suzuki, et al., 2004; Verhagen, et al., 2002; Yang, et al., 2003). Generally speaking, this protein appears to exert similar functions as Smac/Diablo (second mitochondria derived activator of caspases / Direct IAP binding protein with low pI). In a caspase-independent and Apaf1-independent fashion, Omi/HtrA2 is able to induce cell death using its serine protease activity (Hegde, et al., 2002; Suzuki, et al., 2004). Structural analyses showed that cytosolic Smac/Diablo is a symmetric homo-dimer (Chai, et al., 2000). After the release from the mitochondria Smac/Diablo binds IAPs in the cytosol. In this way, Smac/Diablo regulates IAPs by inhibiting their activity (Vaux and Silke, 2003). Therefore, it amplifies caspase activity, since IAPs inhibit the activity of processed caspases (Du, et al., 2000).

2.5 Crosstalk between ER and mitochondria

The mechanisms and the part of crosstalk between the mitochondria and the endoplasmic reticulum are not entirely illuminated. But it seems that cytochrome *c* induced apoptosis activated by ER - mitochondria crosstalk is important for ER stress mediated cell death (Momoi, 2004). Moreover, the main signal in ER-mitochondria crosstalk is thought to be calcium. After activation of the death receptor pathway and consequent activation of caspase-8, BAP31 (Bcl-2-associated protein 31) is cleaved to a p20 fragment. BAP31 is an integral ER membrane protein that seems to be a mediator of crosstalk between the two organelles and it has pro-apoptotic capacities. It is cleaved and activated by a unique isoform of caspase-8 (Breckenridge, et al., 2002). The p20 cleavage product of BAP31 causes the release of Ca^{2+} from the ER. Liberated Ca^{2+} is taken up by the mitochondria inducing the recruitment of Drp1. Drp1 mediates the scission of the outer mitochondrial membrane, resulting in dramatic fragmentation and fission of the mitochondrial network and cytochrome *c* release (Breckenridge, et al., 2003). Calcium signals from the ER regulate the opening of the permeability transition pore. Absorbed Ca^{2+} in the matrix causes at a certain level the opening of the mitochondrial transion pore, which leads to loss of the mitochondrial membrane potential and hence to the release of cytochrome *c*

and apoptotic factors. This process is followed by translocation of cytochrome *c* to the ER, where it interacts with IP_3 receptors to induce a positive feedback loop (Boehning, et al., 2003). Recent studies of the ER-mitochondrial communication presented some evidence for regulation of the IP_3 receptor-mediated Ca^{2+} release by both pro- and anti-apoptotic proteins. Pro-apoptotic factors have been described to facilitate the mobilization of ER Ca^{2+} thereby strengthening the calcium signal propagation to the mitochondria. This may lead to Ca^{2+}-dependent mitochondrial membrane permeabilization (Hajnoczky, et al., 2006). ER membrane proteins also interact with Bcl-2 proteins and thereby influence the apoptotic events. Further, it was reported that Bax and Bak induced cytochrome *c* release by interacting with VDAC. With its cytosolic domain BAP31 can interact with procaspase-8, Bcl-2 and Bcl-x_L, but the BH3-only protein Spike can block the formation of the BAP31 – Bcl-x_L complex (Mund, et al., 2003). Another BH3-only protein, Bad, might also be involved in ER activation. It is dephosphorylated by calcineurin, a Ca^{2+}/calmodulin dependent protein phosphatase and induces the release of pro-apoptotic factors from the mitochondria (Wang, et al., 1999).

2.6 Caspases

Caspases (cysteine-aspartate proteases) are key proteins in the apoptotic process. They specifically cleave their substrates following an aspartate residue (Earnshaw, et al., 1999). Caspases are synthesized as catalytically inactive pro-caspases i.e. zymogens. They need an apoptotic stimulus to be activated by either proteolytic cleavage by other caspases or by autocatalysis to become an active enzyme (Cohen, 1997; Shi, 2002). The caspase family comprises in human 11 members (13 in mammalian). Based on observations made in C. elegans, the first member of this family was described in 1992 as the CED-3 homologue interleukin-1β-converting enzyme (ICE, later caspase-1) (Cerretti, et al., 1992; Thornberry, et al., 1992). According to there structure, several other caspases were identified. The amino acid sequence QAC(R/Q/G)G, found in the active site of caspases is highly conserved (Alnemri, et al., 1996). The substrate recognition motif varies among caspases, making them target-specific substrates (Thornberry, 1997). Nevertheless, all caspases cleave at the peptide bond C-terminal of aspartate residues. Zymogens consist of 3 domains, one N-terminal pro-domain, followed by one large (~20kDa) and one small subunit (~10kDa), which are separated by linkers. After proteolytic cleavage both subunits form heterodimers, made of 2 subunits of each proform, with a catalytic site. Caspases can undergo autoactivation or be activated by other caspases.

2.6.1 Initiator and executioner caspases

Caspases can be divided into two groups (figure 4), the initiator caspases (caspase-2, -8, -9, -10) and the executioner caspases (caspase-3, -6, -7) (Thornberry and Lazebnik, 1998). These two groups differ in the length and structure of their prodomain. Initiator caspases carry a long prodomain with a DED and a CARD (caspase recruitment domain) domain and are the link between cell signalling and apoptosis. The CARD domain is binding the adapter molecules, the DED cares for the hydrophobic binding of the adapter protein (Earnshaw, et al., 1999). Caspases-8 is the main mediator in the extrinsic cell death pathway induced by the TNF family members. Binding of death ligands (CD95, Fas) leads to trimerization of the death receptors and aggregation of death domains (DD). Together with FADD (Fas associated death domain) which recruits procaspase-8 the death-inducing signalling complex (DISC) is formed, where caspase-8 is activated (Muzio, et al., 1996). Also in the intrinsic cell death pathway the formation of a complex, the apoptosome, consisting of procaspase-9 and its adapter protein Apaf-1 is required. The pro-domain of the initiator caspase interacts with the adaptor protein leading to dimerization and finally to autocatalytic activation (Earnshaw, et al., 1999). Autoproteolytic cleavage yields in two small and two large subunits forming an active caspase as a homodimer of two heterodimers (Nicholson, 1999).

The effector caspases share a short pro-domain and act downstream of the apoptotic pathway. Initially, they are cleaved by the initiator caspases at a specific asparagin residue, followed by autoproteolytic cleavage. Active effector caspases cleave substrates escalating the death signal and executing apoptosis (Savill and Fadok, 2000). Caspase-3 is the main effector caspase that cleaves the majority of the cellular substrates in apoptotic cells. Caspase-7 is highly similar to caspase-3 and has comparable substrate specificity (Degterev, et al., 2003). They both amplify mitochondrial caspase activation signalling (Lakhani, et al., 2006).

The position of caspases in the pathway is not just defined by their structure and length, but also by their subcellular localization (Zhivotovsky, et al., 1999).

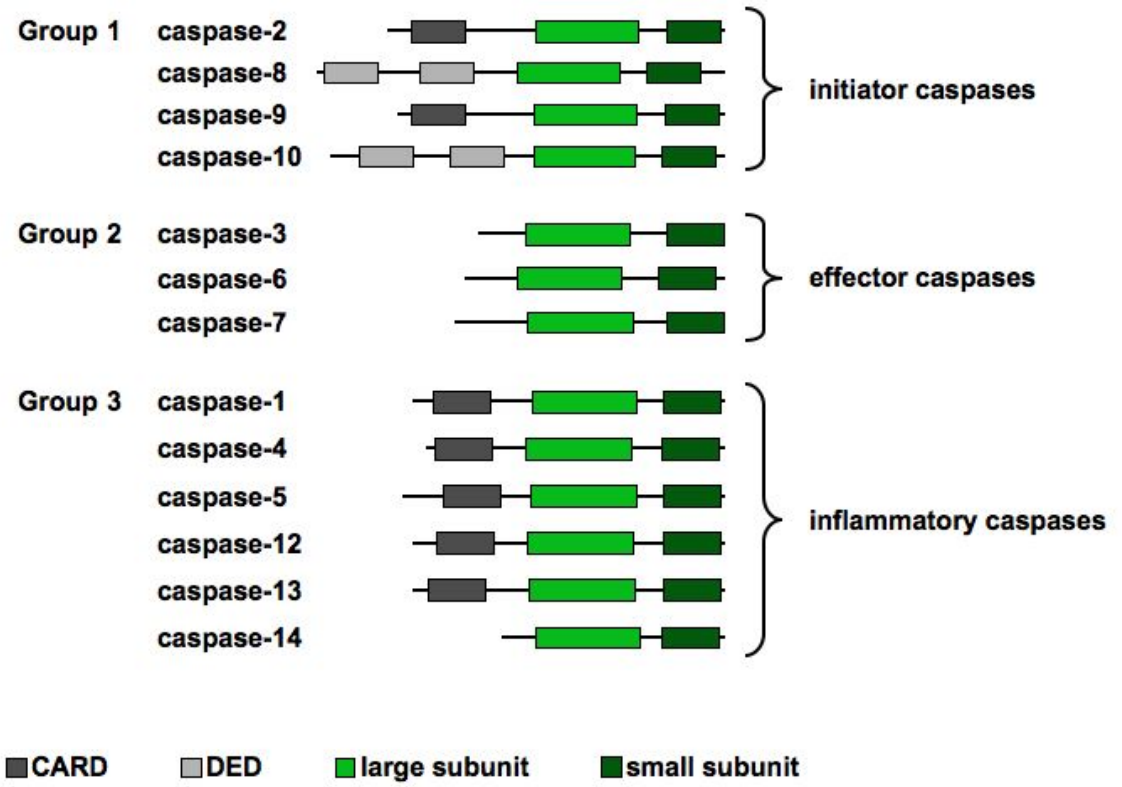

Figure 4: The caspase family

There are three major groups of caspases, group 1: initiator caspases, group 2: effector caspases and group 3: inflammatory caspases. Caspases are synthesized as pro-caspases with an N-terminal pro-domain. The active caspase is a heterotetramer of two large and two small subunits.

2.6.2 Substrates of the caspases

Caspases target substrates, which are either maintaining the metabolism of the cells or proteins involved in the apoptotic machinery (Fischer, et al., 2003). Caspases cleave different substrates in a different manner. However, processing of the substrate can be directly connected to the morphological changes during apoptosis. In the final steps of apoptosis the cell loses its shape. This is indicated by caspases that degrade structural proteins, which maintain e.g. the cytoskeleton (Brockstedt, et al., 1998). Among them are actin filaments and nuclear lamins, which are cleaved by caspase-6 (Orth, et al., 1996). Caspases cleave proteins involved in cell cycle regulation and DNA repair (Cohen, 1997). In healthy cells, ICAD (inhibitor of caspase-activated DNase) is in a complex with the inhibitor DFF45. Activation requires cleavage of the inhibitor protein by caspase-3 resulting in active CAD-nuclease which then translocates to the nucleus (Enari, et al., 1998; Sakahira, et al., 1998). PARP is a nuclear enzyme and is activated upon cleavage by caspase-3 at an early stage of apoptosis. It is cleaved into a small, N-terminal fragment and a large, C-terminal (catalytical) subunit abrogating its DNA repair and ATPase activity. PARP catalyzes the transfer of ADP-ribose polymers to other nuclear proteins, which are

in involved in DNA repair and stabilization. If caspases are not active, PARP is excessively consuming energy during the apoptotic process and cells end up dying by necrosis (Kaufmann, et al., 1993). Thus, it seems that PARP may act as a molecular switch between apoptosis and necrosis (Fischer, et al., 2003).

2.7 The Bcl-2 family

The members of the Bcl-2 (B-cell lymphoma (gene) 2) family are important regulators of apoptosis. They transmit external survival or death signals inside the cell. The Bcl-2 family members are grouped into two subfamilies of anti-apoptotic and pro-apoptotic proteins (figure 5). They all share at least one of the four conserved α-helical so-called Bcl-2 homology (BH) domains (BH1, BH2, BH3, BH4). These domains are conserved motifs (Cory and Adams, 2002). The pro-apoptotic proteins involve a subgroup, which comprises the BH3-only proteins (Huang, et al., 2002). The anti-apoptotic members share all four BH domains, whereas the pro-apoptotic members do not a possess BH4. Instead, they are subdivided into the multi-domain group carrying BH1-3 and the BH3-only group. BH1 to BH3 are responsible for binding by forming a hydrophobic pocket. They give the proteins the ability to form homo- and heterodimers (Borner, 2003). Anti-apoptotic proteins prevent permeabilization of the mitochondria, which is on the other hand promoted by the multi-domain group of the pro-apoptotic proteins. Some of the anti-apoptotic proteins interact directly with members of the pro-apoptotic group (Gross, et al., 1999; Rosse, et al., 1998). The balance in-between anti-apoptotic and pro-apoptotic proteins decide about life or death of the cell.

Members of the Bcl-2 family can regulate apoptosis in different manner: by forming protein channels in mitochondrial membranes or by changing the membrane structure by interactions with lipids. They regulated ion fluxes and release of other proteins by the permeabilization of the mitochondrial membrane and the endoplasmic reticulum (Sharpe, et al., 2004).

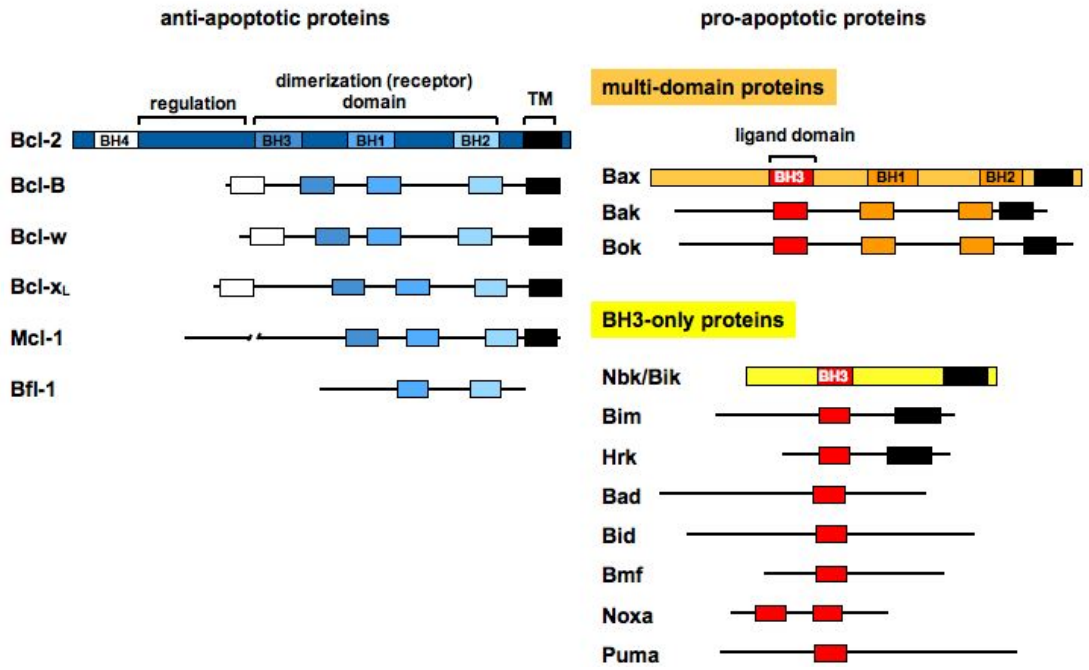

Figure 5: The Bcl-2 family

Bcl-2 family members possess at least one of four BH (Bcl-2 homology) domains and are grouped according to their ability to inhibit or activate cell death. The pro-apoptotic proteins bind with their BH3 domain to the hydrophobic pocket formed by the BH1-BH3 domains of the anti-apoptotic proteins. The C-terminal transmembrane (TM) region is a hydrophobic, single membrane spanning alpha helix that mediates localization to intracellular membranes.
***Anti-apoptotic members** of the Bcl-2 family*
***Pro-apoptotic members** of the Bcl-2 family, subgrouped into multi-domain proteins and BH3-only proteins.*

2.7.1 The anti-apoptotic proteins

The anti-apoptotic members of the Bcl-2 family share three or four Bcl-2 homology (BH) domains, which are essential for apoptosis (figure 5). Proteins such as Bcl-2, Bcl-x_L (Boise, et al., 1993), Bcl-w (Gibson, et al., 1996), A1/Bfl-1 (Choi, et al., 1995; Lin, et al., 1996) and Mcl-1 (Kozopas, et al., 1993) belong to this group. Bcl-2 and Bcl-x_L possess all four BH domains, where as other anti-apoptotic family members such as Mcl-1 carry only BH1 and BH2. The BH-domain serves as binding site for interaction with other proteins. BH1, BH2 and BH3 form a hydrophobic groove, which is stabilized by the N-terminal BH4-domain (Huang, et al., 2002) . This groove represents the binding site for the BH3 α-helix of the BH3-only proteins (Sattler, et al., 1997). This interaction neutralizes the anti-apoptotic family members. In healthy cells, the anti-apoptotic proteins primarily might hold back Bax and Bak from disturbing intracellular membranes, especially the outer mitochondrial membrane (Liu, et al., 2003). Anti-apoptotic proteins can become pro-apoptotic after N-terminal proteolytic cleavage of the BH4 domain (Cheng, et al., 1997). Furthermore, their anti-apoptotic effect seems to depend solely on their BH4 domain, since its deletion led to loss of their activity (Huang, et al., 1998). With their C-terminal tail

the anti-apoptotic proteins such as Bcl-2, Bcl-w, Bcl-x_L and Mcl-1 are able to insert themselves into sub cellular membranes, including the outer mitochondrial membrane, the endoplasmic reticulum or the nuclear envelope (Cory and Adams, 2002; Duriez, et al., 2000). High expression of Bcl-2 and Bcl-x_L is found in several types of cancer. Bcl-2 is an integral membrane protein, even in healthy cells, whereas Bcl-w and Bcl-x_L only incorporate into membranes after death signals. Bcl-2 and Bcl-x_L inhibit apoptosis by insertion into the outer mitochondrial membrane, they may maintain the membrane integrity (Sharpe, et al., 2004). Bcl-x_L was reported to inhibit cytochrome *c* release (Daniel, et al., 2003). There is some discussion about how and if the anti-apoptotic members are involved in mitochondrial membrane permeabilization. Structural features seem to make it likely that they have pore forming abilities, since they show similarities with several bacterial toxins, colchicines A, E1 and diphtheria toxin (Antonsson, et al., 1997; Minn, et al., 1997; Schendel, et al., 1997; Schlesinger, et al., 1997). Others say that VDAC is involved in the regulation of the anti-apoptotic group members. VDAC is located at the outer mitochondrial membrane. Interaction of VDAC and the anti-apoptotic proteins causes the exchange of anions between cytosol and the intermembrane space of the mitochondria (Heiden, et al., 2000). Moreover, Bcl-2 and Bcl-x_L keep pro-apoptotic BH3-only proteins inactive by binding to them in a complex.

Bcl-2, Bcl-x_L and Bcl-w strongly inhibit apoptosis in response to many, but not all, cytotoxic insults. Every cell requires protection by at least one anti-apoptotic family member, as they are chief guardians of tissue homeostasis (Cory and Adams, 2002). Efficient apoptosis calls for neutralization of several anti-apoptotic proteins and imply that not all the anti-apoptotic proteins may have the same function (Adams and Cory, 2007).

2.7.2 The pro-apoptotic proteins

The pro-apoptotic family members are subdivided in multi-domain proteins and BH3-only proteins. Pro-apoptotic proteins Bax (bcl-2 associated protein X) (Oltvai, et al., 1993), Bak (Chittenden, et al., 1995) and Bok (Hsu, et al., 1997) contain BH1-BH3 but lack BH4 and are therefore also called multi-domain proteins. In healthy cells Bak is held in an inactive monomeric state in the outer mitochondrial membrane through its association with VDAC2 (Cheng, et al., 2003). Bax is a monomeric cytosolic protein, inactive through interactions with several proteins (Breckenridge and Xue, 2004). Upon activation they both undergo a conformational change. First Bax has to translocate to the mitochondria (Hsu, et al., 1997; Wolter, et al., 1997) before inserting into the outer membrane (Goping, et al., 1998), whereas Bak stays in the mitochondrial membrane, where they both oligomerize (Antonsson, et al., 1997). Their carboxyl-terminus is essential for targeting to

mitochondria. One model is that Bax and Bak form pores to release pro-apoptotic factors from the mitochondria. The other hypothesis says that they associate with parts of the permeability transition pore (Cory and Adams, 2002). But both models agree on permeabilization of the outer mitochondrial membrane by Bax and Bak. And both multi-domain proteins stimulate the release of cytochrome *c* form the mitochondria using the voltage-dependent anion channel (VDAC) (Daniel, et al., 2003). Bax and Bak are indispensable for apoptotic signalling, since double knockout experiments showed that development and proliferation is not possible without these proteins, either Bax or Bak has to be present in order to fulfil the assignment properly (Lindsten, et al., 2000). Cells without Bax and Bak are resistant to most apoptotic stimuli (Cheng, et al., 2001). Bax and Bak mainly regulate the intrinsic pathway, being localized at the mitochondria, but they also operate at the ER (Scorrano, et al., 2003; Wei, et al., 2001). Currently two models are discussed: 1. Direct activation, holds that certain BH3-only proteins, termed activators, Bim and tBid, can bind to Bax and Bak directly and promote their activation, in this model, the remaining BH-only proteins, termed sensitizers, bind only to the pro-survival proteins and purportedly act by displacing any bound Bim or tBid, allowing them to directly activate Bax and Bak. 2. Indirect activation, on the other hand, suggests that all the BH3-only proteins engage only their pro-survival relatives and act by preventing them from countering Bax or Bak activation, on this model, Bim and tBid are potent inducers of apoptosis simply because they can engage all the pro-survival proteins (Willis and Adams, 2005; Willis, et al., 2007). In the extrinsic pathway, truncated Bid initiates oligomerization of Bax or Bak leading to cytochrome *c* release from mitochondria (Wei, et al., 2000), since cells lacking both of these proteins did not undergo apoptosis after Bid activation. But if at least one of these two multi-domain proteins is present, cell death will be initiated (Wei, et al., 2001). Interestingly, high levels of anti-apoptotic proteins block Bax oligomerization and pore formation, but no Bcl-2 - Bax complex could be detected (Mikhailov, et al., 2001). The BH3-only group comprise quite a lot of members, such as Bid (Wang, et al., 1996), Noxa (Oda, et al., 2000), Puma (Nakano and Vousden, 2001; Yu, et al., 2001), Bim (O'Connor, et al., 1998), Nbk (Boyd, et al., 1995; Han, et al., 1996), Bad (Yang, et al., 1995) and some more (figure 5). The BH3 motif is a short sequence of nine amino acids and the only commonality of these proteins. The BH3-only proteins function as sensors of apoptotic stimuli and are charged to trigger apoptosis (Huang and Strasser, 2000). The crucial decision on life or death seems to be fought on these membranes, although most family members are recruited to these sites upon apoptotic signal (Adams and Cory, 2007). In healthy cells the BH3-only proteins are in an inactive state (Huang and Strasser, 2000). Only upon apoptotic signal they are activated and perform their duty. Distinct apoptotic stimuli activate different proteins of this group, which then deliver the death

signal to the mitochondria by engaging Bax/Bak or Bcl-2/Bcl-x_L (Puthalakath and Strasser, 2002). For example, Noxa and Puma are induced by transcription with p53 as their transcription factor. P53 is activated upon DNA-damage, irradiation and cytotoxic drugs (Han, et al., 2001; Lakin and Jackson, 1999). Other proteins of the BH3-only group are activated by posttranslational modifications, e.g. Bad, which is dephosphorylated (Harada, et al., 1999). Bid is activated by proteolytic cleavage. In its inactive form Bid is a cytosolic protein, but once it is cleaved, truncated Bid (tBid), it translocates to the mitochondria (Li, et al., 1998). There, it stimulates the permeabilization of the mitochondrial membrane and therefore the release of apoptotic factors. Expression of several BH3-only proteins, such as Bim, Bad and Bid in Bax/Bak double knockout cells could not induce apoptosis, suggesting that BH3-only proteins require Bax or Bak to mediate apoptotic signals (Zong, et al., 2001). Most members have additionally a hydrophobic sequence at the C-terminus, which helps them to integrate into organelle membranes.

Bid is activated upon cleavage by caspase-8 after initiation of the extrinsic apoptotic pathway (Li, et al., 1998). Normally, Bid is localized as an inactive form in the cytosol. After activation, truncated Bid (tBid) translocates to the mitochondria to enhance the apoptotic signal (Gross, et al., 1999). Additionally, Bid activates Bax by inducing a conformational change, followed by the translocation of Bax to the mitochondria (Eskes, et al., 1998). Truncated Bid seems to amplify the perturbation of the mitochondria by forming homotrimers in the membrane (Cory and Adams, 2002). Apart from amplification of the mitochondrial pathway, tBid can also convey death signals form other organelles. Bid has an unique place in apoptosis, since it connects the extrinsic and intrinsic pathway (Daniel, 2000). The BH3-only protein Nbk/Bik localizes at the ER and not at the mitochondria, it mediates the activation of the mitochondria in a Bax-dependent manner (Gillissen, et al., 2003). Puma and Noxa and Hrk/DP5, are controlled primarily at the transcriptional level. Puma and Noxa are expressed upon p53 activation and they were shown to cause outer membrane permeabilization. Co-immunoprecipitation studies showed that Noxa binds to Bcl-2 and Bcl-x_L, but not to Bax (Oda, et al., 2000).

If the balance between anti-apoptotic and pro-apoptotic Bcl-2 family members is shifted in the favour of the pro-apoptotic proteins, they bind to and occupy the anti-apoptotic proteins, thereby liberating Bax and Bak (Gogvadze and Orrenius, 2006).

2.7.2.1 Bim

This BH3-only protein is subject to this thesis and will therefore be introduced in more details. While screening for proteins that bind to Bcl-2, a novel protein, named Bim (Bcl-2 interacting mediator), was discovered (O'Connor, et al., 1998). The only common feature

with other known proteins was its BH3 domain, placing it into the BH3-only group of the Bcl-2 family. Bim plays a major role in embryogenesis, in the control of haematopoietic cell death and as a barrier against autoimmunity (Bouillet, et al., 1999). Originally three different Bim splicing variants were found (O'Connor, et al., 1998), but there are also additional splicing variants, all encoded by the same gene (figure 6). These various isoforms differ in size and apoptotic strength. The best characterized are the three main isoforms Bim_{EL}, Bim_L and Bim_S is constitutively pro-apoptotic and appears to be the most toxic, whereas Bim_{EL} and Bim_L are held in an inactive form in healthy cells. Binding to the dynein motor complex light chain LC8 (DLC1) of the microtubule sequesters Bim_{EL} and Bim_L. In response to cytokine removal, calcium flux, microtubule perturbation (by taxol a microtubule polymerizing drug) or cellular damage by UV-irradiation, Bim_{EL} and Bim_L are released from the microtubule in a complex with LC8 (Puthalakath, et al., 1999). The LC8-Bim complex can bind to and antagonize the anti-apoptotic proteins (Borner, 2003) and is recruited to the mitochondria. There are controversial reports on Bim_S about its function and expression. Tissue distribution studies for Bim demonstrated expression of Bim_{EL} and Bim_L in haematopoietic, epithelial, neuronal and germ cells, while Bim_S was not detected by Immunoprecipitation (IP) or Western blot analysis. It was speculated that Bim_S is only expressed in specific cells that need to be extinguished quickly or that Bim_S is not expressed under physiological conditions (Bouillet, et al., 1999; Puthalakath, et al., 1999). On the other hand, there are data showing expression of all three isoforms at similar levels in DLD-1 and HSC-2 cells (Adachi, et al., 2005). Besides, colony-forming assays and cell death studies illustrated the differences of all three isoforms in their cytotoxicity. Bim_S was found to block colony formation and to stimulate apoptosis in the most effective way (O'Connor, et al., 1998).

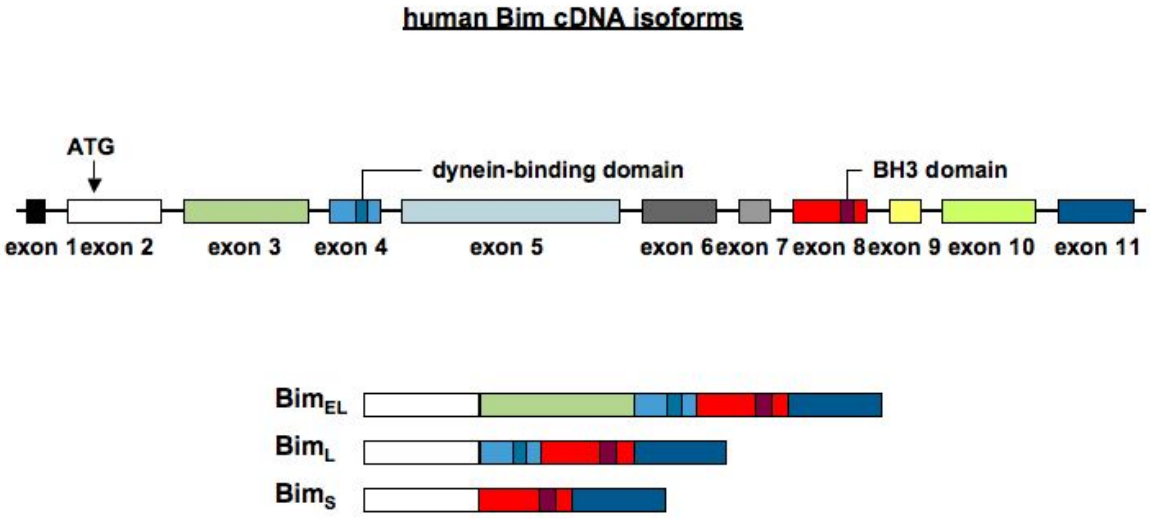

Figure 6: The three main isoforms of Bim

Schematic structures of the three main isoforms are shown. Bim_{EL} and Bim_L contain the dynein binding site by which they are attached to the microtubule, Bim_S lacks this region.

Bim is regulated by transcriptional induction since upregulation results in an elevated amount of Bim-Bcl-x_L complexes (Bouillet, et al., 1999) as well as by phosphorylation by c-Jun N-terminal kinase (JNK) (Lei and Davis, 2003) and is subject to several types of post-translational modifications (Puthalakath, et al., 1999). The BH3-region is essential for the interaction of Bim with anti-apoptotic Bcl-2 family members and for most of its deadly effect. Its hydrophobic C-terminus enables Bim to localize to cytoplasmic membranes (O'Connor, et al., 1998). Furthermore, it cannot trigger cell death in *bax/bak* double knockout cells. Hence, it might be that multi-domain pro-apoptotic proteins are activated by their release from anti-apoptotic factors upon BH3-only protein interference (Cheng, et al., 2001). Bim seems to act upstream of Bax and Bak, since this BH3-only protein was not able to induce neither cytochrome *c* release nor apoptosis in cells deficient for Bax and Bak (Wei, et al., 2001). Being at the microtubule, Bim has the perfect place to serve as stress sensor and communicator of stress signal to Bax and Bak. Overexpression of Bim highlighted its cytotoxity in several cell types, although it was shown that a few molecules of Bim are enough to induce apoptosis even in the presence of increased levels of anti-apoptotic proteins (Strasser, et al., 2000). Indeed, Bim possibly is the most potent killer of any other Bcl-2 family protein. Also, to date, Bim is not only the sole BH3-only protein expressing three different splicing products, but all of these main isoforms interact with Bcl-2 and Bcl-x_L.

3 Materials and Methods

3.1 Cell lines

Prostate carcinoma sarcoma DU145 cells were purchased from ATCC (Manassas, USA. This cell line was isolated from a brain tumour of a patient with metastatic prostate cancer by K R. Stone (Stone et al., 1978). The cells carry a mutation for p53 as well as for Bax.

DU145Bax cells were generated as described earlier (Hemmati, et al., 2002) in the laboratory using retroviral infection. Briefly, for expression of Bax in the Bax-negative DU145 cells we employed the retroviral vector HyTK-Bax (Weinmann, et al., 1997), which contains the human Bax-a cDNA under the control of a CMV promoter. Supernatant of the virus producing packing cell line Fly-18 (Cosset, et al., 1995) was used to infect DU 145 cells in the presence of 8mg/ml polybrene (Sigma Chemical Co., Grunwald, Germany). Hygromycin (0.5 mg/ml) was added to the cells for selection 48 h later. After 3 weeks of selection the cells were subcloned. Bax expression of the clones was determined by Western blot analysis as described below.

DU145Bak cells were generated as described earlier (Radetzki, et al., 2002). Briefly, the human Bak cDNA was cloned by PCR amplification from human cDNA using electroporation. Stable transfectants were selected in 1mg/ml G418. Mock transfectants were generated by the use of the empty pcDNA3 plasmid.

DU145 EGFP-Bax and EGFP-Bak cells were generated as described earlier (von Haefen, et al., 2004) by transfection using Lipofectamine 2000 (Invitrogen Gibco, Karlsruhe, Germany). cDNA of Bak and Bax were amplified with PCR. PCR products were digested and cloned as BglII/EcoRI fragments into the corresponding sites of the vector pEGFP-C2 (BD Biosciences Clontech, Heidelberg, Germany) to obtain Bax or Bak that carry the EGFP tag at the N-terminus. Stable transfectants were selected by the use of G418 at 1 mg/ml, subcloned and EGFP-Bak or EGFP-Bax expression of the clones was determined by fluorescence microscopy.

Media: DMEM high glucose (4.5g/l) medium supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin and 100µg/ml streptomycin (All from GICO, Karsruhe, Germany).

HEK293 cell line is a transformed primary human fetal kidney cell line, which constitutively expresses AdE5E1 proteins (Graham et al. 1977). For this reason they were used for

adenoviral amplification and titration.

Media: DMEM high glucose (4.5g/l) medium supplemented with 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin.

3.2 Storing stock cells

For constituting stocks of different cell lines, 1x106 cells in 500µl of pure media were supplement with 500µl of a 1:5 DMSO:FBS mix in cryotubes, frozen for 2h at –20°C and then stored at –80°C and subsequently stowed in liquid nitrogen.

3.3 Agarose Electrophoresis and extraction of DNA

The separation of the DNA bands was performed with 1% agarose gels containing 0.1µg/ml of ethidiumbromid. The agarose gel was run in a BioRad gel chamber with 1xTBE running buffer.

DNA was extracted from the agarose gel with QIAEX II gel extraction kit (Qiagen, Hilden, Germany). The appropriate DNA-band was cut from the gel and was weighted. The gel slice was incubated with 3 times volumes of buffer QX 1 followed by addition of 30µl of QIAEX 2 buffer containing silica beads. The mixture was incubated for 10min at 50°C to allow the DNA to bind to the beads. Next, the solution was centrifuged at 14000rpm for 30 sec and the pellet was washed twice with 500µl of PE buffer. The pellet was air-dried and the DNA was eluted with water.

The concentration of the DNA was determined by photometric measurement at 260nm while water served as a standard.

10x TBE: 108g Tris
55g boric acid
7.44g EDTA
in 1l aqua dest.

3.4 PCR

DNA was amplified by the polymerase-chain-reaction. The mixture contained 10ng of DNA, 1pmol of each primer, 200µM dNTPs, 10x buffer and 1U Taq polymerase in a total volume of 20µl (all from Invitek, Berlin, Germany).

To create the DNA fragments the following program was used: 95°C for 10min to activate the polymerase, 25 cycles of, denaturation of the DNA at 95°C for 60s, annealing at 58°C for 60s, elongation at 72°c for 90s.

3.4.1 Primers

E1A sense: 5' – GAG ACA TAT TAT CTG CCA CGG AGG – 3'
anti-sense: 5' – TTG GCA TAG AAA CCG GAC CCA AGG – 3'
(Adesanya, et al., 1996)

E4 sense: 5' – GTA GAG TCA TAA TCG TGC ATC AGG – 3'
anti-sense: 5' – TTT ATA TGG TAC CGG GAG GTG GTG – 3'
(Adesanya, et al., 1996)

Bim sense: 5' – GGA TCC ATG GCA AAG CAA CCT TCT – 3'
anti-sense: 5' – ACC TCT TAC GTA ACT ACG AGA TCT – 3'

All primers were purchased from BioTez GmbH, Berlin, Germany

3.5 Precipitation of DNA

1:10 volumes of (3.5M) potassium acetate and 2x volume of 100% ethanol was mixed with the DNA and incubated at -20°C for 15min. After that, the mixture was centrifuged at 14000rpm for 30min at 4°C. The pellet was washed 3x with ethanol, dried and finally dissolved in water. 1 OD equals to 50mg/ml of DNA.

3.6 Ligation of DNA

DNA fragments were ligated into plasmids by using T4 DNA ligase. Up to 200ng of DNA were incubated with 50ng of the appropriate vector together with 1U of T4 ligase in the provided 1x ligase buffer over night at 14°C.

3.7 Transformation of bacteria by heat shock

50µl of heat competent E.coli DH5α bacteria (Clontech, Saint-Germain-en-Laye, France) were incubated with 5µl of DNA on ice for 30min. Then, the bacteria-DNA mix was "heat-shocked" for 90s at 42°C followed by incubation for 5min on ice. 500µl of LB-media was added to the mixture and incubated for 1h at 37°C. Finally, the bacteria were smeared on an agar plate, containing 50µl/ml ampicilin. The plates were incubated over night at 37°C.

LB Medium:	10 g Trypton
	5 g yeast extract
	5 g NaCl
	in 1 l aqua dest.
Agar plates:	15 g Agar
	1 l LB-Medium
	100 µg/ml ampicillin

3.8 Immunochemistry

$1.5x10^5$ cells were plated on sterile cover slips placed into 6-well plates and infected with adenoviral vectors at 25 MOI after 24h. After three times of washing with PBS, the cells were fixed with ice-cold 1% paraformaldehyde for 30min. The fixed cells were permeabilized with ice-cold 100% methanol for 1min. The cells were again washed with PBS and incubated with blocking solution for 30min at RT. The primary antibodies were diluted in blocking solution, applied on the cells and incubated over night at 4°C, followed by secondary antibodies for 1h at RT. Finally, the slides were washed with PBS and mounted in Dako fluorescent mounting medium (DakoCytomation, Glostrup, Denmark) and dried at 4°C over night.

To stain the mitochondria, the cells were incubated with MitoTracker Green (Molecular Probes, Leiden, the Netherlands) at 37°C for 1h with a final concentration of 500nM.

The nuclei were stained with DAPI (Sigma Aldrich, Munich, Germany) at a final concentration of 0.5µg/ml.

3.9 Western blot analysis

3.9.1 Preparation of protein samples and determination of the concentration

Cells were harvested by trypsinization and centrifuged at 2000rpm, 4°C for 5min. Pellets were washed with ice cold PBS and lysed in an appropriate amount of lysis buffer on ice for 30min. After centrifugation at 14000rpm, 4°C for 15min the supernatant was collected as protein sample. To separate the cytosolic from the organelle fraction, the pellets were resuspended with a hypotonic lysis buffer and immediately centrifuged at 2000rpm, 4°C for 5min. the supernatant was kept as the organelle fraction and the pellet was incubated in lysis buffer on ice for 30min and treated as described above. To detect cytochrome *c* release, the cells were separated into a cytosolic and mitochondrial fraction by using "mito buffer", a specific lysis buffer. Cell pellets were incubated with the "mito buffer" on ice for 5min followed by centrifugation at 2000rpm, 4°C for 5min. the supernatant containing the mitochondrial fraction was kept and subjected to determination of protein concentration as described above. The remaining pellet was incubated with lysis buffer on ice for 30min. the pellets were centrifuged at 14000rpm, 4°C for 15min the supernatant was collected as protein sample representing the cytosolic fraction.

The protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, USA). 10µl of protein was mixed with 200µl of BCA solution (reagent A: reagent B, 50:1) in a 96-well plate and incubated at 37°C for 30min in the dark. The absorption was measured at 620nm. A BSA concentration row served as standard. The protein concentration was calculated based on the standard curve.

Lysis buffer	2mM EDTA
	0.1% SDS
	1% Triton-X100
	1mM Na3VO4
	1mM beta-glycerolphosphate
	1 tablet protease inhibitor cocktail ("Complete", Roche Diagnostics)

Hypotonic buffer:	200mM HEPES pH 7.4
	10mM KCl
	2mM $MgCl_2$
	1 mM EDTA
	in 100ml
	to 1ml of buffer add prior to use:
	7.5µl 100mg/ml digitonin
	7.5µl 10mM PMSF

3.9.2 SDS-PAGE

SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is a method to separate proteins and peptides according to their molecular weight (Laemmli, 1970). The anionic detergent SDS denaturates proteins by wrapping its hydrophobic tail around the polypeptide backbone, giving the protein a negative charge, which is proportional to its length. The reducing agent β-mercaptoethanol in the sample buffer cleaves any disulfide bonds between cysteine residues, resulting in a completely unfolded protein. Like this, the original conformation and charges of the proteins do not have any influence on how they migrate through the gel. The only parameter is their molecular weight. The proteins are concentrated at the end of the stacking gel before entering the separating gel. Electrophoresis was carried out with a Bio-Rad Mini-PROTEAN® 3 system. Gels were prepared with 0.75 mm spacers and 10 or 15 pockets.

Samples were mixed with 5x sample buffer, boiled for 5min at 95°C and equal amounts of protein (25µg) were loaded on a 14% SDS-PAGE gel. Electrophoresis was performed at 120V for the first 5 minutes and continued with 180V for approximately 1h.

Separating gel	(14%) aqua dest
	1.5M Tris/HCl, pH 8.8
	actrylamide/bisacrylamide solution (29:1; 40%)
	100µl 10%APS
	100µl 10%SDS
	4µl TEMED

Stacking gel	2.185ml aqua dest.
	380µl 0.5M Tris/HCl, pH6.8
	375µl actrylamide/bisacrylamide solution (29:1; 40%)
	30µl 10%APS
	30µl 10%SDS
	3µl TEMED
5x sample buffer	260 mM Tris/HCl, pH 6.8
	12.5% mercaptoethanol
	20% Glycerol
	2% SDS
	0.01% Bromphenolblue
5x running buffer	100mM Tris
	1M glycin

3.9.3 Blotting of the proteins onto nitrocellulose membrane

Electrophoretically separated proteins were transferred onto 0.2µm nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by semi-dry blotting using a Bio-Tad Trans-blot SD transfer cell (Bio-Rad, Munich, Germany). Membranes and filter paper (Schleicher & Schuell GB005, Dassel, Germany) were kept in CAPS buffer for 3min prior to use. 3 layers of filter followed by the membrane, SDS-gel and again 3 layers of filters were placed on the anode and finally covered with the cathode. Blotting was performed at 1mA/cm^2 for 1h. Homogenous transfer was verified with ponceau S red staining.

Blotting buffer	10mM CAPS, pH 11
	10% (v/v) methanol
Ponceau	0.1% ponceau-S
	in 5% acetic acid

3.9.4 Immunodetection

The blotted membranes were blocked for 1h in blocking buffer to avoid unspecific binding of the antibodies and to avoid background staining. Next, the membranes were incubated with primary antibodies diluted in blocking buffer for 1h at RT. After washing three times with blocking buffer, the membranes were incubated for one hour with horseradish peroxidase-conjugated secondary antibodies, diluted in blocking buffer. Finally, the membranes were washed three times with PBST and protein bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate system (Pierce, Rockford, USA). This system is based on the chemical reaction of luminol with peroxidase and hydrogen peroxide. The light emission emerging of this reaction is captured on film by autoradiography. The membrane was incubated in the ECL solution for 3min (Luminol:peroxide buffer, 1:1) and exposed to a hyperfilm ECL-film (Amersham Biosciences, Buckinghamshire, England) for an appropriate time.

Blocking buffer	10% casein (Roche, Mannheim, Germany)
	0.1% Tween
	in PBS
PBST	0.1% Tween
	In PBS
	0.5% SDS

3.9.5 Antibodies

Primary

- polyclonal rabbit anti-Bim (Cell Signaling Technology, Beverly, MA, USA)
- clone 7H8.2C12 mouse anti-cytochrome *c* (PharMingen, Heidelberg, Germany)
- polyclonal goat anti-Caspase-3 (R&D Systems, Wiesbaden, Germany)
- polyclonal goat anti-Caspase-9 (R&D Systems, Wiesbaden, Germany)
- monoclonal mouse anti-Caspase-8 (Cell Signaling Technology, Beverly, MA, USA)
- polyclonal rabbit anti-Mcl-1 (Santa Cruz Biotechnologies, Heidelberg, Germany)
- polyclonal rabbit anti-Bak-NT (Upstate, Charlottesville, VA, USA)
- polyclonal rabbit anti-Bax-NT (Upstate, Charlottesville, VA, USA)
- monoclonal mouse anti-Bcl-2 (Novocastra Laboratories, Newcastle, UK)

- polyclonal rabbit anti-PARP (Cell Signaling Technology, Beverly, MA, USA)
- monoclonal mouse-anti BiP (PharMingen, Heidelberg, Germany)
- monoclonal mouse anti-CHOP (Alexis Corp., Lausen, Switzerland)
- monoclonal rat anti-BAP31 (Apotech, Epalinges, Switzerland)
- polyclonal rabbit anti-β-Actin (Sigma-Aldrich, Munich, Germany)
- Alexa Fluor 594 anti-rat IgG (Molecular Probes, Leiden, The Netherlands)
- Alexa Fluor 488 (Molecular Probes, Leiden, The Netherlands
- Mito Tracker Green FM (Molecular Probes, Leiden, The Netherlands)

Secondary

- HRP-conjugated anti-mouse IgG (Promega, Mannheim, Germany)
- HRP-conjugated anti-rabbit IgG (Promega, Mannheim, Germany)
- HRP-conjugated anti-goat IgG (Santa Cruz Biotechnologies, Heidelberg, Germany)

3.10 The Adenoviral vector

The shuttle plasmid pHVAd2-TRE is used to insert the gen of interest into the E1 region of the adenovirus type 5. It consists of 2.6kb and a multicloning site as described in Bett et al., 1994. After modification by Gillissen at al., 2003 this shuttle plasmid additionally contains a CMV_{mini} promoter and a BGH-poly A signal. The tTA expression cassette from pTet-Off (BD Biosciences Clontech, Palo Alto, USA) was first cloned as XhoI-PvuII fragment into pHVAd2 shuttle vector upstream of CMV_{mini}. Bim cDNA fused to an N-terminal myc tag was cloned into pSL1180 (Amersham Pharmacia Biotech, Freiburg, Germany) and then inserted as a BamHI-XbaI fragment into the pHVAd2 vector resulting in TRE-myc-Bim. Finally, the Ad5 vector was fused to TRE-myc-Bim by homologous recombination of pAd-tTA creating pAd5-myc-Bim-tTA.

3.10.1 Homologous recombination

Adenoviral vector was completed by homologous recombination of pHVAd1 containing the adenovirus genome and the shuttle vector pHVAd2 containing tTA in Escherichia coli strain BJ5183 RecBC-sbcB. Transformation of the bacteria was achieved by electroporation, after which the bacteria were grown of agar plates containing ampicilin. Colonies were picked and grown in liquid LB-media also containing ampicilin and

incubated at 37°C over night. The adenoviral vector was isolated from the colonies and was verified by digestion with the appropriate restriction enzymes. E.coli DH5α bacteria were transformed with the accurate plasmid for multiplication. Finally, the adenoviral vector was isolated from the bacteria and used for transfection of HEK293 package cells.

3.10.2 Calcium-phosphate transfection

10µg of PacI digested adenoviral DNA were mixed with 25µl of 2.5M $CaCl_2$ and adjusted with water to a total of 250µl. 250µl of transfection buffer were added drop-by-drop to the mix and gently shaken. Upon shaking calcium-phosphate crystals are formed that bind DNA. The total of 500µl was incubated for 30min at RT. Different concentrations were added to HEK293 cells incubated in 1ml serum free medium. After 1.5h 1ml media with 20% FBS was added to the cells. Once plaques were visible in cell layer, the cells were harvested and the crude virus lysate (CVL) was used for virus amplification.

Transfection buffer:
8g NaCl
370mg KCl
250mg $Na_2HPO_4x2H_2O$
5g HEPES
in 500ml water
pH 6.75
sterilize

3.10.3 Amplification of the adenovirus

HEK293 cells were plated into 50 150cm^2 flasks. 24h hours later the culture media was discarded and 10ml suspension containing CVL and serum free fresh media was added to the cells. After incubation of 1.5h an equal volume of 10ml media with 20% of FBS was added to the cells. Once the cells detached from the flask, showing sign of viral infection, cells and media were collected and centrifuged. 35ml of CVL and the pellet were kept and the rest of the supernatant discarded.

3.10.4 Adenovirus purification

Before starting the purification, the CVL was thawed and frozen three times. 2.5ml of 1.25g/cm^3 CsCl were poured into an Ultra-Clear centrifuge tube (Beckman Instruments GmbH, Munich, Germany) and 2.5ml of 1.5g/cm3 CsCl were slowly added beneath. Then,

7 ml of CVL from the virus purification was added onto this CsCl gradient. Next, the gradient was centrifuged (Beckmann Optima LE 80-K) at 30 000rpm for 2h. After centrifugation 2 bands were visible in the gradient. The virus band (the lower one) was harvested using a 1ml syringe and mixed with 1.35g/cm^3 CsCl to a final volume of 3ml. Then, 3ml of 1.35g/cm3 and subsequently 3ml of 1.25g/cm^3 CsCl were carefully added creating a gradient. This was followed by over night centrifugation at 30 000rpm resulting in a purified virus band on the gradient. The virus was collected as previously with a 1ml syringe.

A NAP-25 column (Amersham Buchler, Braunschweig, Germany) was washed 4 times with 5ml of adenovirus suspension buffer. 1ml of the virus, gained from the gradient, was applied onto the column. Then, the column was washed with 1.5ml adenovirus suspension buffer. Finally, 2ml of adenosuspension buffer was added to the column and collected into a fresh tube. The purified virus solution was stored as 50µl aliquots at -80°C.

Adenoviral suspension buffer:	135mM NaCl
	3mM KCl
	1mM $MgCl_2$
	100mM Tris/HCl
	sterilize
	add 10% glycerol

3.10.5 Virus titration

A dilution series starting with 1µl, which was cut in half in each step, was pipetted into a 24well plate using serum free media for dilution. HEK293 cells were plated into a 24well plate and infected with the pre-prepared dilution series of the virus by using one well per dilution rate in a volume of 500µl. After 1.5h incubation time, 500µl of media containing 20% FBS was added to the cells. Approximately 14 days after infection the plaques of each well were counted. The titer was estimated based on the plaque formation and the dilution rate.

3.10.6 Verification of replication competent adenovirus (E1a-PCR)

For verification of the adenovirus, the E1a region was tested for unwanted recombination. During virus amplification in HEK293 cells the E1a region could be inserted into the adenoviral vector by homologous recombination and enables the virus to replicate.

Therefore the adenoviral DNA was amplified for its E1a region by PCR. Detection of the E1a DNA band would make the adenovirus unusable. For purification of viral DNA 1µl of the purified virus was mixed with 20µl of proteinase K-Mix and incubated for 90min at 55°C. 300µl of Phenol/Chloroform/Isoamylalkohol pH 8.0 (ratio 25/24/1) was added to the sample. After vortexing the sample was centrifuged for 5min at 14000rpm. The upper phase was transferred into a new tube and mixed with 300µl of chloroform. This step was followed by centrifugation for 5min at 14 000rpm. The upper phase containing the DNA was transferred into a new tube and the DNA was acquired by precipitation (see 3.4).

The acquired DNA was tested for E1a positivity by PCR and bands were detected by agarose gel electroporation. As a positive control, PCR and gel electroporation were performed for the E4 region, which is part of the adenoviral vector.

Proteinase K-Mix:	5mM EDTA
	20mM Tris/HCl, pH 8.0
	0,2%(v/v) SDS
	0.25µg/µl proteinase K

3.10.7 Infection of the cells with adenovirus

All media was removed from the cells to be infected and replaced with media without FBS containing the adenovirus at the indicated amounts of MOI. After incubation for 1.5h at 37°C, the same volume of media with 20% FBS was added to the cells.

3.11 Flow cytometry

Measurements with FACS (fluorescence-activated cell sorter) allow detection of single cells according to different parameters, such as size, granularity and density. Cells floating in a sheath liquid are hit by a laser beam (argon ion laser, excitation at 488nm). For each cell passing, it is measured how much light is absorbed (forward scatter, shows the relative size) and reflected (side scatter, represents the relative density and is proportional to the cell granularity). Moreover, different fluorescence emitted by the cells can be quantified in different channels (FL1=537nm, FL2=597nm, FL3=650nm) of the flow cytometer.

Evaluation of the obtained data was displayed by using histograms. They show the fluorescence intensity versus the cell numbers.

Flow cytometry analysis were performed with FACScan (Becton Dickinson, Heidelberg, Germany) and later evaluated with CellQuest analysis software.

3.11.1 Measurement of apoptosis

One of the typical features of apoptosis is the fragmentation of the DNA, which often includes also the disruption of the nucleus. The cell partially loses its DNA since the cell membrane is getting permeable during apoptosis. So it is known that apoptotic cells have a lower DNA content than normal cells in the G1-phase. Thus, this DNA can be detected as a so-called sub G1 peak and is a marker for apoptotic cells.

Propidium iodide which intercalates with DNA is used to measure the increased fluorescence of its fluorochrome. The intensity represents the DNA-content of the cell.

After infection with $AdBim_L$ or $AdBim_S$ $2x10^5$ cells were harvested by trypsinisation at the indicated time points. The cells were fixed on ice in a 2% formaldehyde solution for 30min. After centrifugation for 5min at 1300rpm 50µl ice cold PBS was added to the each sample, followed by 100µl ethanol (-20°C). The samples were kept on ice for 15min. After centrifugation for 5min at 1300rpm the cells were resuspended in PBS containing 40µg /ml DNase-free RNase A (Roche Molecular Biochemicals, Mannheim, Germany) and incubated for 30min at 37°C. Finally, the samples were pelleted and resuspended in 100µl PBS containing 50µg/ml propidium iodide (Sigma, Deisenhofen, Germany) and subjected to analysis in the FL3-channel.

3.11.2 Annexin-V FITC

To distinguish apoptosis from necrosis, cells were stained with Annexin-V-FITC (fluorescin isothiocyanate) and counterstained with propidium iodide (PI). Annexin-V-FITC binds to phosphatidylserine (PS) on the outer side of the plasma membrane. Propidium iodide can only enter cells with a disrupted plasma membrane. Thus, PI and Annexin-V positivity is a sign for late apoptosis or necrosis, whereas cells positive for Annexin-V, but negative for PI are generally defined as early-apoptotic (Vermes et al. 1995, 1997).

After infection with $AdBim_L$ or $AdBim_S$ cells were collected at indicated time points. Cells were washed twice with cold PBS and resuspended in Annexin binding buffer at 1 x 10^6 cells/ml. Next, 2 µl of Annexin-V-FITC (BD PharMingen, Heidelberg, Germany) and 4 µl PI (20 µg/ml, Sigma-Aldrich, Munich, Germany) were added to 100 µl cell suspension. Samples were incubated for 20 minutes in the dark at room temperature. After incubation, 100 µl Annexin binding buffer was added.

Binding buffer: 10mM HEPES/NaOH pH 7.2
140mM NaCl
2.5mM CaCl2

FITC conjugated peptide carry the corresponding sequence of the active site of the caspase and can therefore bind to the active site.

3.11.3 Measurement of the mitochondrial membrane potential

Intact mitochondria establish a potential in-between the intramembrane space and the matrix created by an electrochemical gradient. Based on this feature of mitochondria kationic and lipophilic dyes like JC-1 (5,5´,6,6´-tetrachloro-1,1´,3,3´-tetraethyl-benzimidazolylcarbocyanin iodide) can accumulate in the matrix. By forming aggregates the fluorescence shifts from green to red. Depolarisation of mitochondria is therefore accompanied by increased green fluorescence and is measured in the FL-2 channel of the FACS.

$2x10^5$ cells were incubated with adenoviral vectors at 25 MOI for the indicated time points. Samples were collected by centrifugation and resuspended in 1ml JC-1 solution (2.5µg/ml PBS). After incubation for 30min at 37°C samples were centrifuged at 1300rpm for 5min at 4°C and washed three times with cold PBS. Finally, the cells were resuspended in PBS and immediately analysed.

3.11.4 Caspase activity

Caspase activity was measured for each caspase individually by using their specific cell permeable caspase inhibitors (FLICA, Kit assay, Serotec, Oxford, England). These peptides bind covalently to cysteine residues and thereby block the activity of the individual caspase. Caboxyfluorescein is attached to the peptide contributing the fluorescence. Binding of the peptide to the corresponding caspase leads to an increase of the fluorescence, which is identified in the FL1-channel. As for the measurement, $2x10^5$ cells were harvested after adenoviral infection at indicated time points and resuspended in 500µl PBS with 30µM final concentration of the peptide. Samples were incubated for 20min at 37°C and washed 3 times with washing buffer. Finally, the cells were diluted in 200µl PBS and measured by FACS.

3.11.5 Caspase inhibition

To inhibit the activity of caspases peptide with a specific sequence are used. They are bound by the active site of the corresponding caspase. For caspase-3 the DEVD-fmk was used, for caspase-9 LEHD-fmk, caspase-8 IETD-fmk (all from Calbiochem, Bad Soden) and for caspase-4 LEVD-fmk (Biovision). To block all the caspases a pan caspase inhibitor zVAD-fmk peptide was used (Calbiochem, Bad Soden).

After infection of $2x10^5$ cells with either $AdBim_L$ or $AdBim_S$, they were treated with the corresponding 20µM caspase inhibitor. The cells were collected at indicated time points and prepared for apoptosis measurement as described in 2.6.1

3.11.6 Calcium release

Thapsigargin is a sesquiterpen-lactone which is extracted form umbellifere Thapsia garganica (Ramussen at al., 1978) Thapsigargin is releasing Ca^{2+} from the ER by specifically blocking SERCA (Ca^{2+}-ATPase of the ER) and none of the other Ca^{2+}-ATPases. Thapsigargin was used as a positive control and subjected to measurement as described.

Fluo-3/AM (Molecular Probes, Leiden, The Netherlands) is an acetoxymethyl ester, which can pass the plasma membrane if it is uncharged. Inside the cells esterases transform it into a charged molecule. In this state the complex binds Ca^{2+} ions, accumulates and becomes fluorescence.

Cells were infected with either $AdBim_L$, $AdBim_S$ or mock treated and cultured for indicated hours. After harvesting $2x10^5$ cells by trypsinization, pellets were resuspended in 500µl fresh media with 10% FBS. Additionally, 10µl of 2mM Fluo-3AM in DMSO solution was added to each sample. Cells were incubated for 45min at 37°C under vigorous shaking and light protection. After the incubation time, samples were centrifuged for 3min at RT and washed three times with PBS. Samples were immediately analyzed by flow cytometry in the FL-1 channel.

3.11.7 Conformational change of Bax and Bak

$2x10^5$ cells were transduced with adenoviral vectors and collected by trypsinization 16h later. After washing with PBS and fixed in 1 ml PBS/0.5% paraformaldehyde (v/v) on ice for 30 min. Again the cells were washed with PBS. N-terminal epitope were detected by conformation specific anti-Bax-NT or anti-Bak-NT antibody (Upstate, Charlottesville, VA, USA). Staining was performed by incubating cells in 1 ml staining buffer (PBS, 1% FCS,

0.1% saponin) containing 0.1 mg of the respective antibody on ice for 30 min. Then, cells were washed in staining buffer, resuspended in 1 ml staining buffer containing 0.1 mg fluorescein-labelled F(ab')2 goat anti-rabbit IgG (H+L) antiserum (Jackson Immuno Research, West Grove, PA, USA) and incubated on ice for 30 min in the dark. After a final washing with PBS the intracellular staining of Bax or Bak was quantified by flow cytometry in the FL1- channel.

4 Results

4.1 Cloning of the adenoviral vector

To clarify the pathway of Bim, full length cDNA of Bim_L and Bim_S was amplified by PCR cloned into an adenoviral vector. The use of an adenoviral expression system gives the advantage to over express the protein of interest at similar levels for each experiment in a panel of cell lines. To regulate the expression of these BH3-only proteins, an inducible adenoviral vector based on the Tet-Off system was established in the group (Gossen and Bujard, 1992). This system comprises all the functional components needed on a single adenoviral vector the gene of interest, its inducible promoter, the region of the transactivator and its constitutive promoter. The basic adenoviral vector is deleted for the E1 and E3 region. The E3 region was replaced by the expression cassette of the tTA-Tet-Off-transactivator and its constitutive CMV-promoter. To create the pAd1-ΔE1/E3-tTA (Gillissen, et al., 2003). The shuttle plasmid pAd2-TRE is used to transfer the gene of interest into the E1 region of the adenovirus type 5. Through modifications, it contains one TRE-element and one BGH-poly(A) signal (Gillissen, et al., 2003). Myc-tagged human cDNA of Bim_L or Bim_S was cloned by PCR into the shuttle vector using specific primers with BamHI and XbaI for restriction site. Bim was inserted into Ad1-tTA by cotransfection of BJ5183 bacteria for homologous recombination of the shuttle vector containing Bim and the adenoviral vector. Therefore, both plasmids the shuttle vector and Ad5-tTA were linearised with PacI. Homologous recombination in these recombinase proficient bacteria resulted in the final adenoviral vectors Ad5-mycBim_L-tTA (Ad-Bim_L) or Ad5-mycBim_S-tTA (Ad-Bim_S). Production of the virus was achieved by transfecting HEK293 cells with the final adenoviral vector.

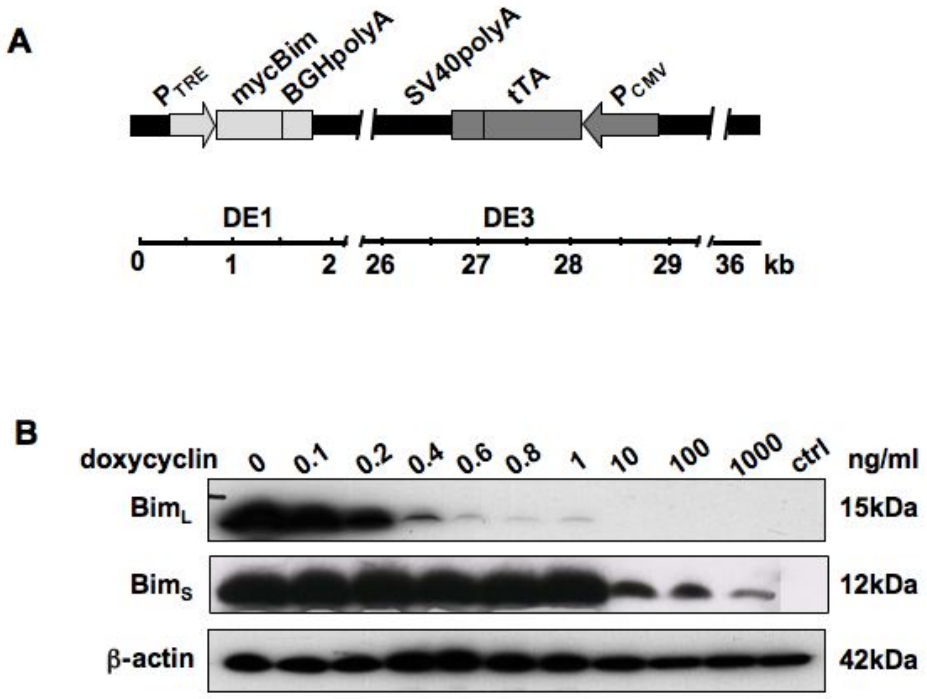

Figure 7: Inducible Bim expression mediated by Ad5-mycBim-tTA

***A**: Genomic structure of recombinant adenovirus Ad5-mycBim-tTA. Ad5 sequences are indicated by black dashes. E1 and E3 regions of Ad5 are replaced by mycBim$_L$ and mycBim$_S$ expression cassette (white boxes), respectively and tTA expression cassette (grey boxes), respectively (P_{CMV}: immediate early promoter of cytomegalovirus tTA tetracycline-controlled (Tet-Off) transactivator; P_{TRE}: tetracycline-responsive element located 5' of the minimal immediate-early CMV promoter).*
***B**: Western blot analysis for Bim$_L$ and Bim$_S$ expression. DU145 Bax cells were transduced with Ad5-mycBim$_L$-tTA or Ad5-mycBim$_S$-tTA and cultured in the presence of different doxycyclin concentrations for 24h.*

4.2 Titration of the adenoviruses

In order to determine the dose and time dependent effect of both adenoviruses, AdBim$_L$ and AdBim$_S$, on cell death, DU145-Bax cells were measured for DNA fragmentation after indicated time points following infection with AdBim$_L$ or AdBim$_S$ at MOIs mentioned. Cells that were not infected with the adenovirus served as a negative control and did not show any sign of apoptosis at any of the time points. Also, when doxycyclin was added to the media no apoptotic cells could be detected showing that this agent is not toxic for the cells (figure 8). After 24h, DU145-Bax cells, which were infected with either AdBim$_L$ or AdBim$_S$ with the indicated MOIs and grown under off conditions showed the same level of apoptosis as the control cells. An apoptotic rate of 4% in the presence of doxycyclin demonstrated that doxycyclin suppressed the expression of Bim in case of both AdBim$_L$ and AdBim$_S$ But once doxycyclin was not present in the media, cell death increased with rising MOIs of each adenovirus. 25 MOI of AdBim$_L$ induced apoptosis in 9% of the cells and 25 MOI of AdBim$_S$ induced 21% (figure 7A). 50 MOI of adenovirus generated 18% of

dead cells after $AdBim_L$ overexpression and 34% after Bim_S overexpression. At 100 MOI the number of dead cells was 33% for $AdBim_L$ and 51% for $AdBim_S$ infected cells. $AdBim_L$ mediated cell death led to 45% apoptosis with 150 MOI and to 57% in cells transduced with $AdBim_S$.

The 48h time point illustrates the strength of $AdBim_S$, since only 25 MOI of this adenovirus provoked 54% of cell death. Increased MOI of 50 ended up with 81% of apoptotic cells (figure 8A). 100 MOI and 150 MOI even induced apoptosis in 88% of the cells. To a lesser extent Bim_L overexpression displayed moderately increasing steps in the apoptotic rate with 12% upon 25 MOI and with 24% upon 50 MOI. 39% and 53% of the cells were killed upon 100 MOI and 150 MOI, respectively. In contrast to $AdBim_S$, the cells infected with $AdBim_L$ and cultured under off conditions at any MOI rate did not present any noteworthy apoptotic rate. Overexpression of Bim_S under off conditions revealed leakiness of the system starting with 50 MOI at 17%, mounting to 22%with 100 MOI and 32% of cell death with 150 MOI.

72h post infection cells visibly showed that the off-system was not able anymore to regulate the suppression of Bim properly (figure 8A). $AdBim_L$, under off conditions, induced only some cell death of 10-15%. DU145-Bax cells infected with $AdBim_S$ under off conditions caused apoptosis in the range of 14% up to 60%. Under on conditions, with 25 MOI of $AdBim_L$ 22% of the cells were found to be apoptotic, with 50 MOI 28%, with 100 MOI 83% and with 150 MOI 89%. $AdBim_S$ demonstrated its full power after 72h by killing nearly all the cells (90%) regardless of the MOI used with the exception of 25 MOI, where it induced 47% of cell death. Of note, cells cultured under off conditions and infected with 50 MOI, 100 MOI or 150 MOI of $AdBim_S$ showed high numbers of dead cells. In consideration of these results, all the experiments were performed with 25 MOI for both, $AdBim_L$ and $AdBim_S$ unless mentioned otherwise. To confirm the expression of $AdBim_L$ or $AdBim_S$ Western blot analyses were performed at four different time points (figure 8B). DU145-Bax cells were transduced with either $AdBim_L$ or $AdBim_S$ and harvested at the indicated time points. As a control, non-infected cells were used that did not show over expression of Bim. DU145-Bax cells, which were transduced with $AdBim_L$ and $AdBim_S$ respectively but grown under off conditions, did not show any Bim expression after 16h. Both isoforms were detected for the first time 6h after infection of the cells with the adenovirus. Later time points displayed the increasing expression for both splicing variants. This Western blot analysis displayed the quick induction of Bim.

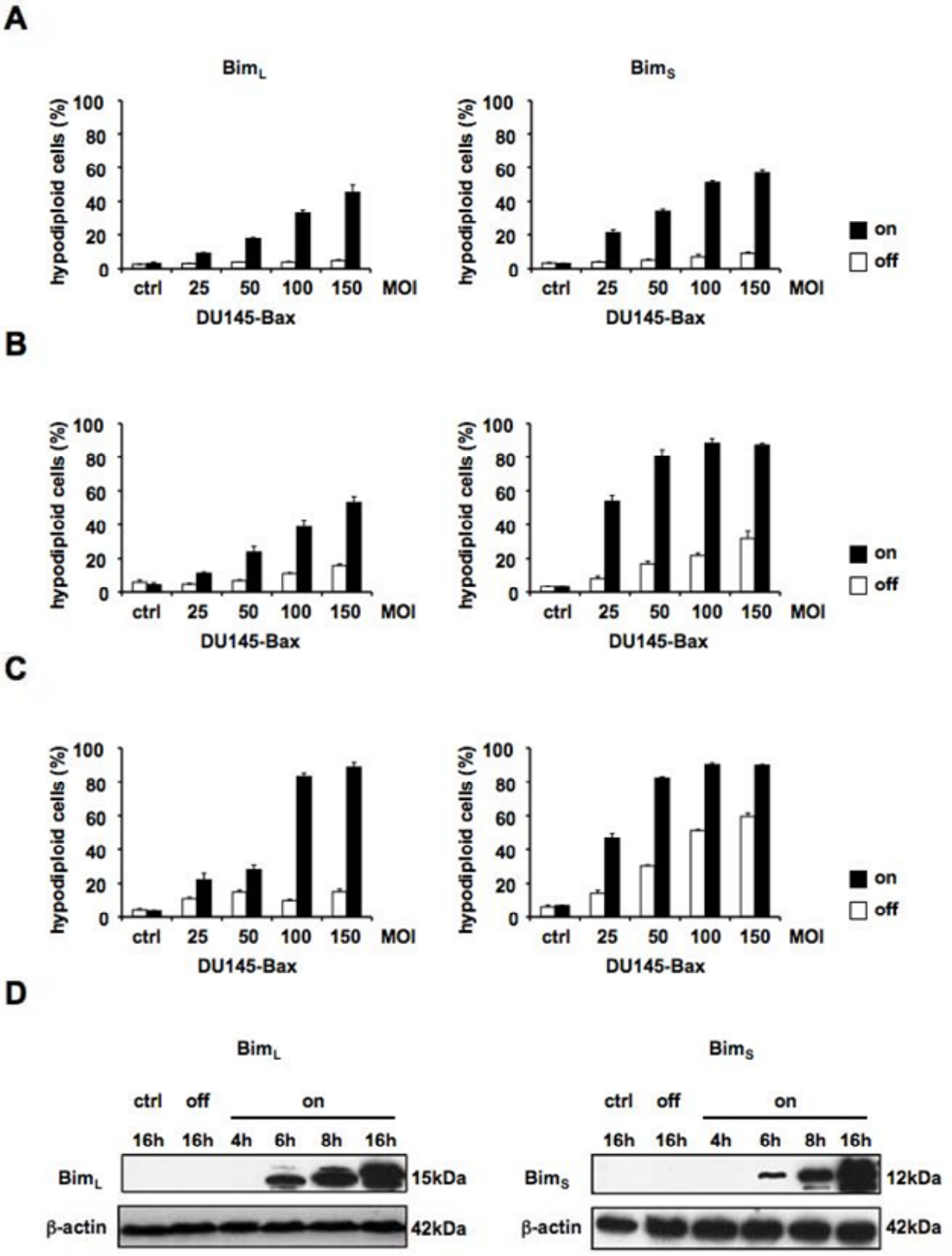

Figure 8: Dose-response for apoptosis induction by Bim

A, B, C*: Left panel: flow cytometric measurement of apoptotic cells with hypodiploid DNA content. DU145-Bax cells were infected with AdBim$_L$ at different MOI and cultured in the presence (off) or absence (on) of doxycyclin for 24h, 48h and 72h (top down).*
Right panel: DU145-Bax cells were transduced with were infected with AdBim$_S$ and treated as described for were infected with AdBim$_L$. Means +/- SD from three independent experiments.
D*: Western blot analysis of DU145-Bax neo cells. Cells were transduced with AdBim$_L$ or AdBim$_S$ and cultured for indicated hours. Cells were harvested after indicated hours and blotted for Bim$_L$ and Bim$_S$, respectively. Actin was used as a loading control.*

4.3 Bim activates the intrinsic mitochondrial pathway

4.3.1 Clustering of Bax and Bak

The multi-domain pro-apoptotic proteins Bax and Bak are known to be involved in the signalling cascade of apoptosis. Once activated these proteins oligomerize at the mitochondria and ensure that the mitochondria are permeabilized by forming pores at the outer membrane.

One way to visualize Bax and Bak clusters at the mitochondria is to make use of green fusion fluorescence proteins. To this end, DU145 cells were stably transfected to express the fusion proteins EGFPBax or EGFPBak, respectively. These cells display green fluorescence even under non-treated (control) conditions (figure 9A and B). Bak is localized to reticular structures since it is associated to the mitochondria (figure 9A). Bax on the other hand is a predominantly cytosolic protein, therefore the fluorescence looks diffuse (figure 9B). The same pictures present themselves under off conditions, when both cell types were infected with the indicated adenovirus, but were cultured in doxycyclin containing media. For both, DU145 EGFPBax and DU145 EGFPBak, regardless whether they are infected with $AdBim_L$ or $AdBim_s$, did not show any Bax or Bak clustering. 24h post infection with $AdBim_L$ or $AdBim_S$ under on condition, the fluorescence microscopy revealed a punctuated pattern of EGFPBak and EGFPBax. Both isoforms of Bim caused clustering of Bak and Bax in consequence of oligomerization of these pro-apoptotic proteins. This demonstrates that Bim activates both pro-apoptotic multi-domain proteins. The pictures in the last row represent cells treated with 1µg/ml of epirubicin as a positive control. Epirubicin is an anthracyclin antibiotic, which acts as a DNA damaging agent by inhibiting topoisomerase II. Therefore it triggers the activation of the mitochondrial pathway, which is marked by the assembly of Bak and Bax oligomers at the outer membrane. DU145 EGFPBax and DU145 EGFPBak cells clearly illustrated clustering of Bax and Bak after treatment with epirubicin.

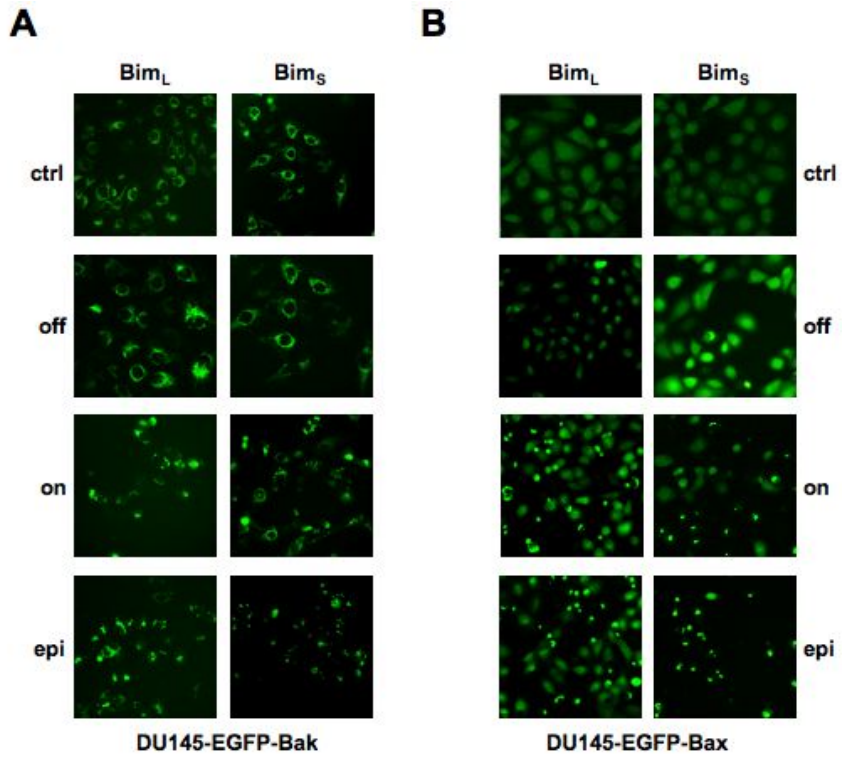

Figure 9: Bim induces oligomerization of Bax and Bak in DU145 cells

***A**: DU145 EGFP-Bak cells were transduced with AdBim$_L$ (left panel) and AdBim$_S$ (right panel) and cultured for 24h in the presence (off) and the absence (on) of doxycyclin or were treated with epirubicin (epi). Pictures were taken with a fluorescence microscope.*
***B**: DU145 EGFP-Bak cells treated as in **A**.*

4.3.2 Re-expression of Bax and overexpression of Bak in DU145 cells

Wild type DU145 are deficient for Bax and express only a small amount of Bak. The two cell types used in the following experiments originate from these cells. DU145-Bax transfectants were generated from mock cells to re-express Bax (figure 10A). Mock transfectants were generated in parallel by use of the empty HyTK vector. DU145-Bak cells overexpress exogenous Bak but do not express Bax, like the DU145 wild type cells. Matching mock transfected cells show a low level of Bak expression (figure 10B). These two cell types, one re-expressing Bax and the other overexpressing Bak, gave the possibility to investigate the interplay of Bim with these pro-apoptotic proteins separately.

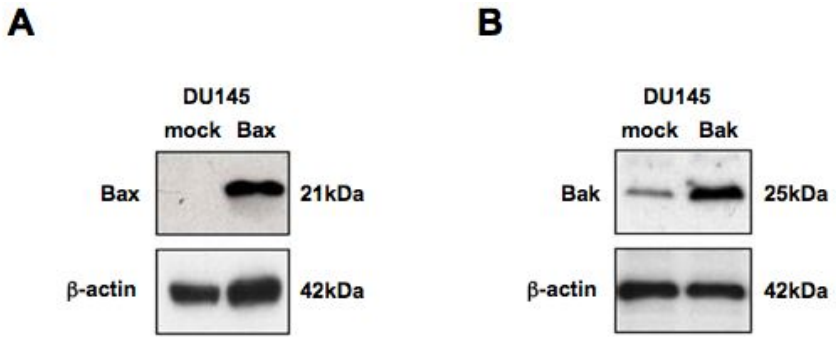

Figure 10: Expression of Bax and Bak in DU145 cells

Western blot analysis of Bax and Bak expression in DU145-Bax and DU145-Bak cells. Non-treated cells were harvested and blotted for Bax and Bak respectively. Actin was used as a loading control.

4.3.3 Conformational switch of Bax and Bak

For further confirmation that Bim activating Bax and Bak, DU145-Bax, DU145-Bak cells and their corresponding mock transfected control cells were transduced with $AdBim_L$ or $AdBim_S$. 16h later, the cells were harvested and stained with conformation specific antibody against the N-terminus of Bax (BaxNT) or the N-terminus of Bak (BakNT). Shift to the higher fluorescence indicating the conformational change of the protein, was determined by flow cytometry (figure 11). First, the cells were examined for a conformational change of Bax. DU145 mock cells, which were not infected with the adenovirus or were grown under off conditions, did not display Bak activation. Also in the corresponding Bax transfectants, no activation could be detected in the control cells or those under of conditions. Overexpression of Bim_L or Bim_S in mock cells, which are Bax negative, showed a similarly low number of cells with BaxNT as the controls, namely 8% for Bim_L and 9% for Bim_S. After $AdBim_L$ expression and under on conditions, in 14% of DU145-Bax cells active Bax was measured. In case of $AdBim_S$, 40% of DU145-Bax cells were stained for Bax conformational change (figure 11A, upper panel). As a control, DU145-Bak and their mock transfectants were investigated for Bax activation, although these cells are Bax negative (figure 11A, lower panel). 4-6% of stained cells positive for BaxNT were detected when the cells were not infected and 8-16% when they were cultured under off conditions. Expression of Bim_L or Bim_S in mock cells yielded for both BH3-only proteins in 11% of cells positively stained for conformational changed Bax. Staining of DU145-Bak cells revealed few positive cells, 13% were found after $AdBim_L$ and 18% after $AdBim_S$ transduction.

Next, the cells were assayed for a conformational change of Bak. Non-treated DU145 mock as well as DU145-Bax cells did not demonstrate a conformational change of Bak. This was also true for cells, which were infected with either of the adenovirus in the

presence of doxycyclin i.e. when the expression was turned off. Only 4-10% of the cells presented staining. Upon Bim_L expression, 13% of DU145 mock cells were detected BakNT positive and 20% upon Bim_S expression (figure 11B, upper panel). 30% of DU145-Bax transfectants expressing Bim_L showed exposure of the BakNT epitope, whereas expression of Bim_S presented 51% cells with positive staining. The same procedure was performed for DU145-Bak cells (figure 11B, lower panel). 4-11% of DU145 mock and 4-16% of DU145 Bak cells, which were control treated or expression of Bim was suppressed, showed BakNT staining. Under on conditions, determination of activated Bak resulted in high rates for both Bim isoforms in these cells. Expression of Bim_L revealed 16% of mock cells with Bak conformational change and expression of Bim_S showed 34%. Staining of DU145-Bak cells displayed 47% positive cells upon Bim_L and 72% upon Bim_S expression. These results confirm that Bim is not only able to activate the multi-domain proteins Bax and Bak but can also trigger apoptosis by both proteins, independently from each other.

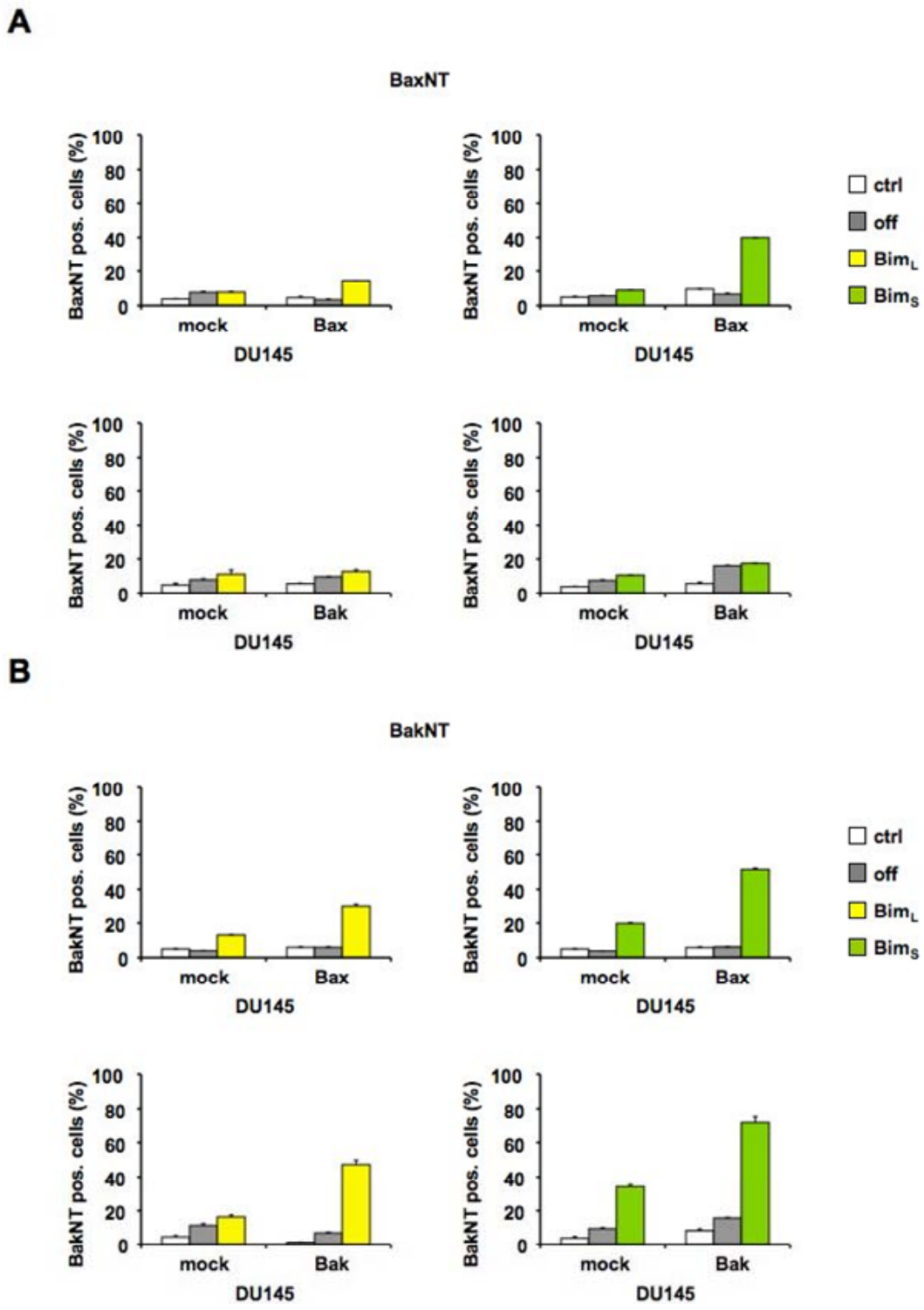

Figure 11: Bim induces conformational change of Bax and Bak in DU145 cells

A: *DU145-Bax and DU145-Bak cells were infected with AdBim$_L$ (left panel) or AdBim$_S$ (right panel) and grown under off or on conditions. After 16h the cells were measured for conformational change of Bax. Means +/- SD from three independent experiments.*
B: *DU145-Bax and DU145-Bak cells were infected with AdBim$_L$ (left panel) or AdBim$_S$ (right panel) and grown under off or on conditions. After 16h the cells were measured for conformational change of Bak. Means +/- SD from three independent experiments.*

4.3.4 Induction of apoptosis by Bim is mediated by Bax and Bak

As shown previously Bim activated Bax and Bak by inducing their conformational change and oligomerization at the mitochondria. The next question addressed was to analyze whether this activation of the pro-apoptotic multidomain proteins is functionally linked to activation of apoptosis and consequently DNA-fragmentation. For this purpose both DU145-Bax and DU145-Bak transfectants were used (figure 12). After adenoviral infection with either $AdBim_L$ or $AdBim_S$ the cells were examined for apoptosis after 48h. Both, Bim_L and Bim_S induced apoptosis in Bax dependent fashion. Also, Bak overexpressing, but Bax negative cells underwent apoptosis (figure 12). In the presence of doxycyclin the expression of Bim was suppressed, thus there was no induction of cell death in the Bax or Bak transfectants or their mock controls. Whereas all control treated cells showed an apoptotic rate of 4-6%, cells under off conditions revealed 14-20% of apoptotic cells. Bim is expressed under on conditions, i.e. when no doxycyclin is present in the medium. Bax overexpressing cells underwent apoptosis at 58% after $AdBim_L$ expression and at 82% after $AdBim_S$ expression. In the mock cells 18% and 26% of apoptosis was found upon overexpression of Bim_L and Bim_S, respectively. This might be due to the fact, that these mock cells express a small amount of Bak, but are Bax negative. The same observation was made for the DU145-Bak mock cells. These cells too express a small amount of Bak. In this case, Bim_L induced 17% and Bim_S 22% of apoptosis. Interestingly, the apoptotic rate of DU145-Bak cells was similar to the ones obtained in the Bax expressing cells. Bim_L caused 58% of apoptosis and 72% of the same cells died after infection with $AdBim_S$. These results suggest that overexpression of Bim can trigger apoptosis via Bax or Bak and that both events are independent from each other. Furthermore, both cell types DU145-Bax or DU145-Bak and even their equivalent mock cells show in all cases a higher apoptotic rate after $AdBim_S$ infection as compared to $AdBim_L$.

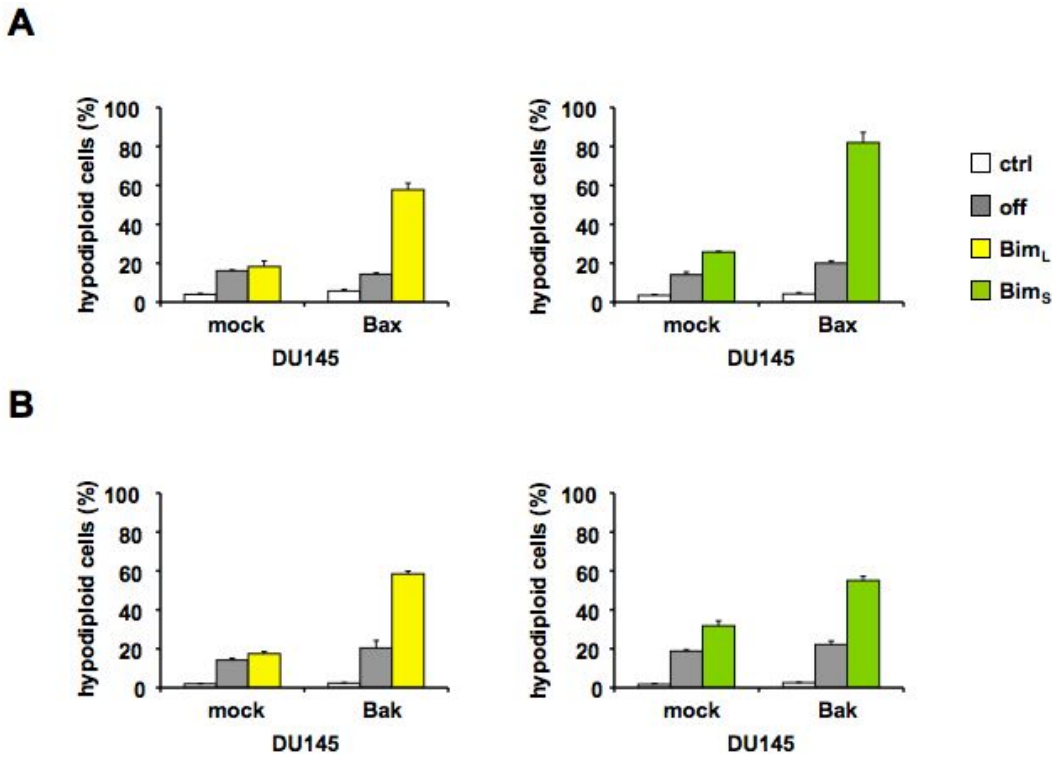

Figure 12: Bim induces apoptosis in a Bax- and Bak-dependent manner

A, B: *Flow cytometric detection of apoptotic cells based on measurement of the cellular DNA content. DU145 cells were transduced with AdBim$_L$ or AdBim$_S$ and cultured in the presence (off) and the absence (on) of doxycyclin. Control cells were mock treated and grown in the absence of doxycyclin for 72h. Means +/- SD from three independent experiments.*

4.4 Activation of pro-apoptotic proteins by Bim leads to the breakdown of the mitochondrial membrane potential

Additionally to the activation of Bax and Bak the integrity of the mitochondrial membrane potential can give evidence to the activation of the intrinsic apoptotic pathway. The cationic carbocyanine dye JC-1 is a fluorescent marker for mitochondrial depolarization during apoptosis. Accumulated JC-1 in the matrix changes its fluorescence emission light from green to red. Cells were infected with AdBim$_L$ or AdBim$_S$, harvested after 24h, loaded with JC-1 and the samples were than analyzed for loss of red fluorescence by flow cytometry (figure 13). Non-treated control cells for both DU145-Bax and DU145-Bak transfectants and their control transfectants did not show any disruption of the mitochondria. The same result was obtained for cells under off conditions. Expression of Bim$_L$ as well as Bim$_S$ led to the breakdown of the mitochondrial membrane potential. Mock showed low mitochondrial membrane potential in 16% of the cells after Bim$_L$ expression and in 34% after Bim$_S$ expression. Bim$_L$ induced reduction of the mitochondrial potential in 32% of DU145-Bax cells and in 63% of the cells after Bim$_S$ expression. DU145-Bak and

the respective mock transfectants displayed a similar rate of apoptosis induction. $AdBim_L$ induced breakdown of the mitochondrial potential in 12% of the mock transfectants and in 30% of Bak overexpressing cells. In 32% of the mock transfectants mitochondrial breakdown of the membrane potential was detected and in 61% of the DU145-Bak cells following Bim_S expression. Thus, Bim triggers the mitochondrial pathway as shown by the decreased numbers of cells with intact mitochondria. Furthermore, Bim_S induced the mitochondrial death pathway more effectively than Bim_L. Moreover, this occurs via either Bax or Bak and this may explain the relatively high rates of mitochondrial membrane potential dissipation and cell death in both mock transfectant types. The very low level at which these cells express Bak is apparently for Bim_S to cause disruption of the mitochondrial membrane potential and mitochondrial permeabilization.

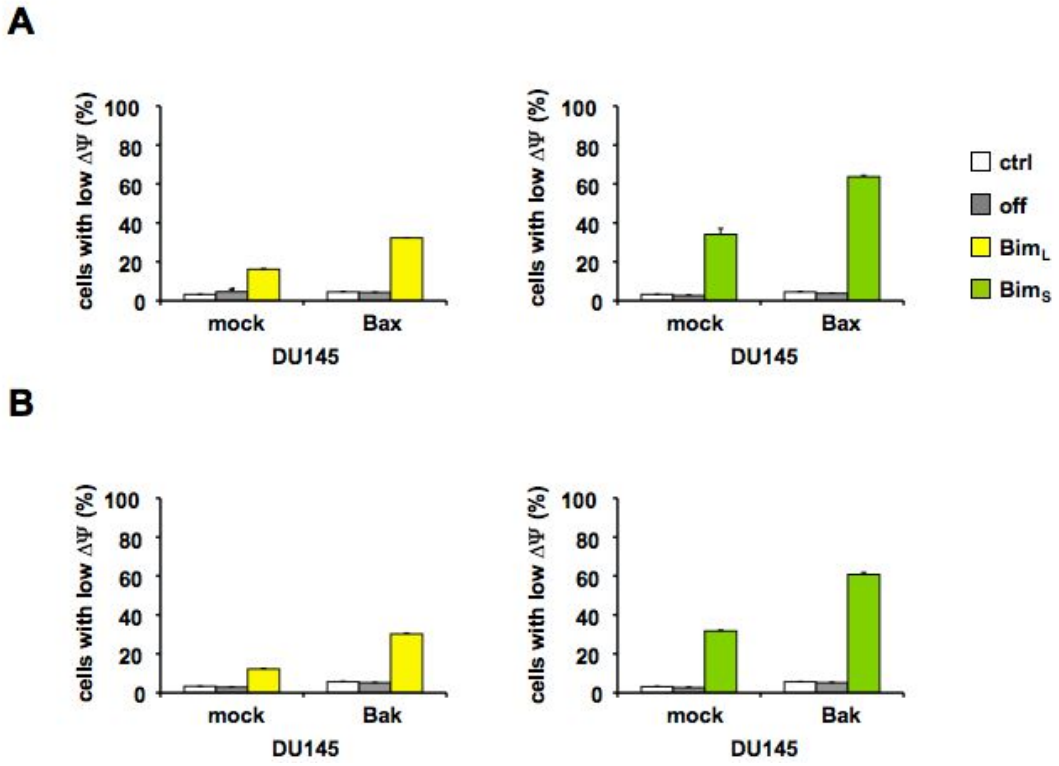

Figure 13: Bim-induced loss of $\Delta\Psi_m$ in a Bax- and Bak-dependent manner

A, B: *Flow cytometric detection of cells with lowered mitochondrial membrane potential. Indicated DU145 cells were transduced with $AdBim_L$ or $AdBim_S$ and cultured in the presence (off) and the absence (on) of doxycyclin. Control cells were mock treated and grown in the absence of doxycyclin. Measurements were performed after 24h. Means +/- SD from three independent experiments.*

Measurement of the mitochondrial membrane potential can give information about the disruption of the outer membrane of the mitochondria. This leads to the release of proteins that are normally harboured in the intermembrane space of the mitochondria, into the cytosol. Additional confirmation that cytochrome *c* release did actually occur was obtained by immunofluorescence staining of cytochrome *c* (Figure 14). Mitochondria were stained with Mito Tracker Red, cytochrome *c* was stained in green and the nuclei were made visible with DAPI staining. DU145-Bax and their mock controls were infected with either $AdBim_L$ or $AdBim_S$ and cytochrome *c* release was determined after 16h. Under off conditions no cytochrome *c* release was visible in neither of the cell types regardless of the virus used. The overlay revealed by the yellow colour that cytochrome *c* is still present in the mitochondria. But expression of Bim in DU145-Bax cells caused liberation of cytochrome *c* as can be seen in the overlay. The higher potency of Bim_S to induce apoptosis is obvious under on conditions in the mock cells. Even with a low level of Bak expression in the mock transfectants, $AdBim_S$ was able to trigger cytochrome *c* release to a noticeable degree.

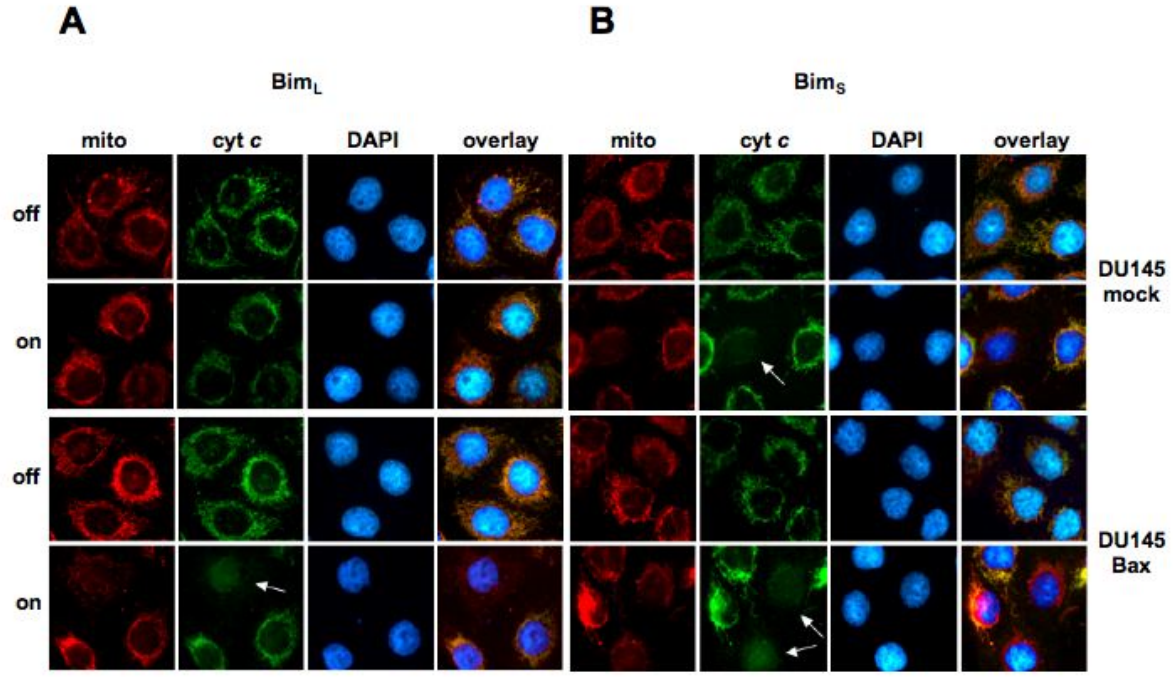

Figure 14: Bim induces cytochrome c release dependent of Bax

A, B: *Immunofluorescence: indicated DU145 cells were transduced with $AdBim_L$ or $AdBim_S$. Cells were stained for mitochondria with MitoTracker Red CMXRos, for cytochrome c with anti-cytochrome c followed by Alexa Fluor 488-conjugated chicken anti-goat antibodies (green fluorescence) and for the nuclei with DAPI staining. Pictures were taken with fluorescence microscope 16h after infection with the corresponding adenovirus.*

4.5 Initiation of the caspase cascade by Bim

The activation of Bax and Bak and the disruption of the mitochondrial membrane are events that take place prior to caspase activation. The following studies aimed to identify the caspases involved in the intrinsic death pathway that is induced by Bim. After adenoviral expression of $AdBim_L$ or $AdBim_S$ and treatment with specific or broad spectrum caspase inhibitors, the apoptotic rate of the cells was determinates by flow cytometry at 48h following transduction. In all experiments the cells were incubated with 20μM of caspase inhibitor (figure 15). Both mock transfectants and likewise DU145-Bax and DU145-Bak, the latter not transduced with the adenoviral vector did not show apoptotic signs, the apoptotic rate amounted to a range of 3-6%. When expression of Bim was suppressed in these cells, 4-8% underwent cell death. In DU145-Bax cells 2-11% of apoptosis was measured, under control or off conditions whereas in DU145-Bak cells 2-7% of the cells died with evidence of genomic DNA fragmentation. In 12% and 6% of the DU145 mock transfectants DNA–fragmentation was detected upon $AdBim_L$ or $AdBim_S$ expression, respectively. Their DU145-Bax counterparts revealed cell death in 33% of the cells after transduction with $AdBim_L$ in the absence of doxycyclin. $AdBim_S$ expression induced apoptosis in 41% of these cells. 35% of the DU145-Bak cells cultured under on conditions were found to be apoptotic following $AdBim_L$ expression, while 41% of the cells died when $AdBim_S$ was expressed. However, in this setting, both isoforms could only trigger 8% of apoptosis in mock cells.

In a first step, using the broad caspase inhibitor zVAD-fmk all caspases were blocked to see if the caspases play functional role in the Bim pathway. Additional treatment with zVAD-fmk reduced apoptosis to 3% in DU145-Bax cells transduced with $AdBim_L$ as compared to Ad Bim_L expression alone in the absence of doxycyclin. This is representative for all non-treated (control) cells and the one under off conditions. In the same setting, also the short version of Bim failed to induce cell death, 6% apoptotic cells were measured. Overexpression of $AdBim_L$ or $AdBim_S$ and addition of zVAD-fmk in DU145-Bak cells led to a similar outcome with 4% of dead cells. To identify the caspases that play a role for Bim triggered cell death caspase inhibitors were employed. The caspase-3 inhibitor zDEVD-fmk caused reduction of apoptosis under on conditions in DU145-Bax cells after $AdBim_L$ expression. Only 17% of the cells died under these circumstances. The same cells overexpressing $AdBim_S$ and grown in the presence of zDEVD-fmk showed 14% of apoptosis. Again, a similar result was found in the DU145-Bak cells: with caspase-3 being blocked, $AdBim_L$ could only induce 20% and $AdBim_S$ 13% of apoptosis. Next, the impact of caspase-9 was examined with the zLEHD-fmk inhibitor. Only 10% of the DU145-Bax cells, which were grown in the presence of the inhibitor went to apoptosis upon expression of $AdBim_L$ and 12% died upon expression of $AdBim_S$.

DU145-Bak overexpressing cells behaved the similarly, presenting also 10% of apoptosis after infection with $AdBim_L$ and 16% with $AdBim_S$. And finally caspase-8 was blocked with zIETD-fmk inhibitor, where a similar effect was achieved. 17% and 12% of dead Bax expressing cells were detected upon Bim_L and Bim_S expression. Their DU145-Bak counterparts reacted the same way after Bim_L or Bim_S expression, namely with 13% of apoptosis.

In general, it can be said, that the specific caspase inhibitors saved around 50% of the cells in both cell types. Thus, the initiator caspases-8 and 9 and the effector caspase-3 are important for Bax and Bak mediated apoptosis upon Bim expression, especially since the pan-caspase inhibitor diminished cell death to the level of not infected cells.

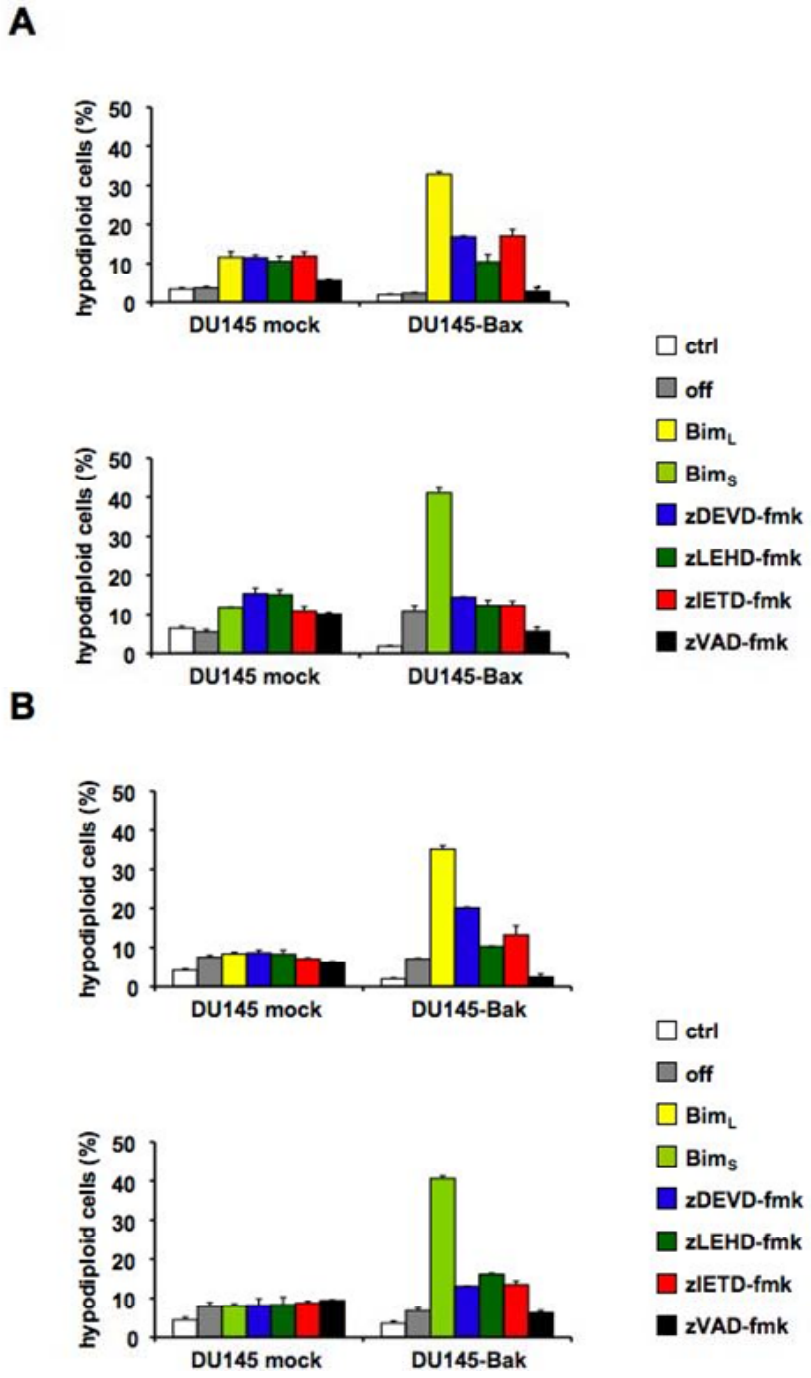

Figure 15: Bim-induced apoptosis is caspase dependent

***A**: DU145-Bax and DU145 mock cells were infected with AdBim$_L$ or AdBim$_S$ in the presence (off) and the absence (on) of doxycyclin and treated with 20μM of the indicated caspase inhibitors. Cells were cultured for 48h and measured for hypodiploid DNA content with flow cytometry. Means +/- SD from three independent experiments.*
***B**: DU145-Bak and DU145 mock cells treated as in **A**. Means +/- SD from three independent experiments.*

Apparently, loss of the mitochondrial membrane potential happens within 24h after Bim expression (figure 13). Once the outer membrane is broken apoptosis associated proteins are released, such as AIF, Smac and cytochrome *c*. These proteins aim to different targets and carry on the apoptotic process. Cytochrome *c* for example is part of the

apoptosome where caspase-9 is activated which in turn leads to the activation of the caspase cascade. Thereby, the pro-form of the caspases is cleaved to become an active caspase. Since Bim is obviously activating the caspase cascade, cleavage products of the caspases should be detectable on the protein level. Hence, total lysates of cells were collected at different time points to perform western blot analysis after infection of DU145-Bax cells with adenoviral Bim. With this time course the generation of the active caspases could be followed (figure 16). As a control, DU145-Bax cells were transduced with $AdBim_L$ or $AdBim_S$ and cultured in the presence of doxycyclin for 24h. Western blot analysis showed that in case Bim is not expressed, there is no caspase activation, cytochrome *c* release or PARP cleavage in these cells (figure 15A, B, first lane). One of the initiator caspases that is activated at early time points of the apoptotic process is caspase-9. Upon Bim_L expression caspase-9 is cleaved after 14h, this time point marks the formation of the apoptosome (figure 16A). 10h after transduction of $AdBim_S$ activation of caspase-9 could be observed (figure 16B). For both isoforms, $AdBim_L$ and $AdBim_S$ the cleavage products could not be detected after 24h, probably due to their degradation. The impact of Bim on the main effector caspase-3 demonstrated the same effect. 14h after expression of Bim_L, pro-caspase-3 was processed to its cleavage products of 20kDa and 18kDa. But at the 24h time point there are no more active caspases detectable by Western blot analyses. Bim_S was more effective by inducing caspase-3 activation already 10h post adenoviral infection. Disruption of the outer mitochondrial membrane potential causes the release of cytochrome *c* and is upstream of caspase activation events. According to the results of caspase activation, cytochrome *c* is detectable in the cytosol (cytosolic fraction) after 14h for $AdBim_L$ and after 10h for $AdBim_S$ expression. PARP (poly-ADP-ribose-polymerase) is a DNA repair enzyme. It is cleaved upon apoptosis for a substrate of caspases-3/7 and is therefore a good marker for this type of caspases mediated cell death. After expression of Bim_L in Bax positive DU145 cells, PARP is cleaved dominantly after 14h and upon Bim_S expression after 10h. Over time the pro-form of PARP disappears and after 18h and 14h respectively, only its cleaved form can be found. The results, gained by this time course, indicate that Bim_S activates the apoptotic pathway faster than Bim_L.

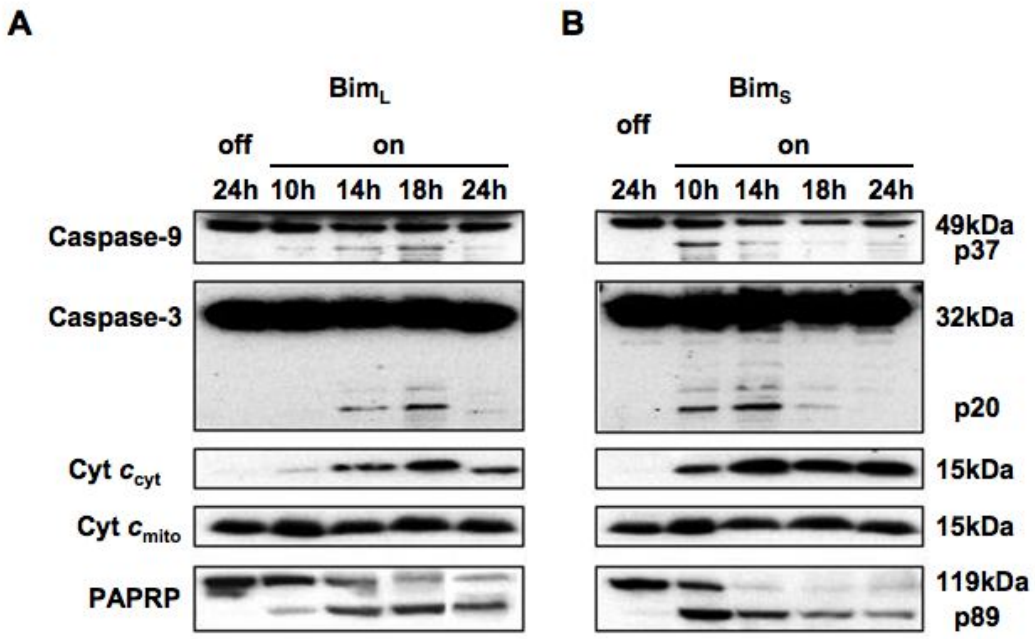

Figure 16: Bim activates proteins of the intrinsic pathway

A: *Western blot analysis for Bim$_L$. DU145-Bax cells were transduced with recombinant adenovirus AdBim$_L$ and cultured for indicated hours in the presence (off) and the absence (on) of doxycyclin.*
B: *Western blot analysis for Bim$_S$, as in **A**.*

4.6 Role of Bcl-2 in Bim induced cell death

Bcl-2 is an anti-apoptotic protein and can antagonize the actions of pro-apoptotic proteins, although the exact mechanism is still strongly debated (Borner, 2002). Therefore, the next step in these investigations was to establish the role of Bim and the potentially involved organelles. The following three kinds of Bax re-expressing DU145 cells were used for the studies. Cells were transfected stably to express Bcl-2 mutants either at the mitochondria (Bcl-2actA) or at the endoplasmic reticulum (Bcl-2cb5). The control cells (neo) were transfected with the empty vector and do not overexpress Bcl-2 (figure 17). To target Bcl-2 to the mitochondria, the membrane anchor was replaced by the mitochondrial insertion signal protein actA of Listeria monocytogenes. To achieve localization of Bcl-2 to the ER, the rat hepatic ER cytochrome b5 isoform targeting sequence was fused to Bcl-2 instead of its C-terminal transmembrane region. The resulting products were transduced into DU145-Bax cells. The purpose of these transfectants was to investigate whether Bcl-2, being an anti-apoptotic protein would be able to protect the cells from Bim-induces apoptosis through a mitochondrial and/or ER pathway.

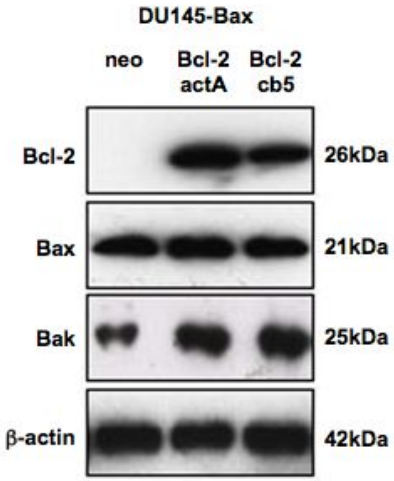

Figure 17: Expression of Bcl-2, Bax and Bak expression in DU145-Bax cells

Western blot analysis of Bcl-2, Bax and Bak expression in DU145-Bax cells.

4.6.1 Bcl-2 targeted to the mitochondria or the ER prevents cell death upon certain apoptotic stimuli

As shown by Western blot analysis, DU145-Bax Bcl-2-transfected cells overexpress Bcl-2 (figure 17). To confirm that these cells express Bcl-2 at the targeted organelle, mitochondria and ER, respectively, immunostaining for subcellular localization was performed. The cells were stained for Bcl-2, mitochondria or endoplasmic reticulum and examined with a fluorescence microscope (figure 18). After fixation and permeabilization the cells were incubated with the primary antibody specific for mitochondria, for the ER or for Bcl-2. Addition of the second fluorescent antibody stained the mitochondria and the ER red or Bcl-2 green. Nuclei were DAPI stained, and thereby visualized in blue. As for the neo control transfected cells there was no staining for Bcl-2 at all, showing that these cells do at best express low levels of Bcl-2 and can be used as a control. DU145-Bax Bcl-2actA cells reveal in the overlay that Bcl-2actA localized at the mitochondria (figure 18, left panel), but was not found at the ER (figure 18, right panel). Vice versa in DU145-Bax Bcl-2cb5 cells, here Bcl-2cb5 inserted into the endoplasmic reticulum (figure 18, right panel), but did not co-localize with the mitochondria (figure 18, left panel).

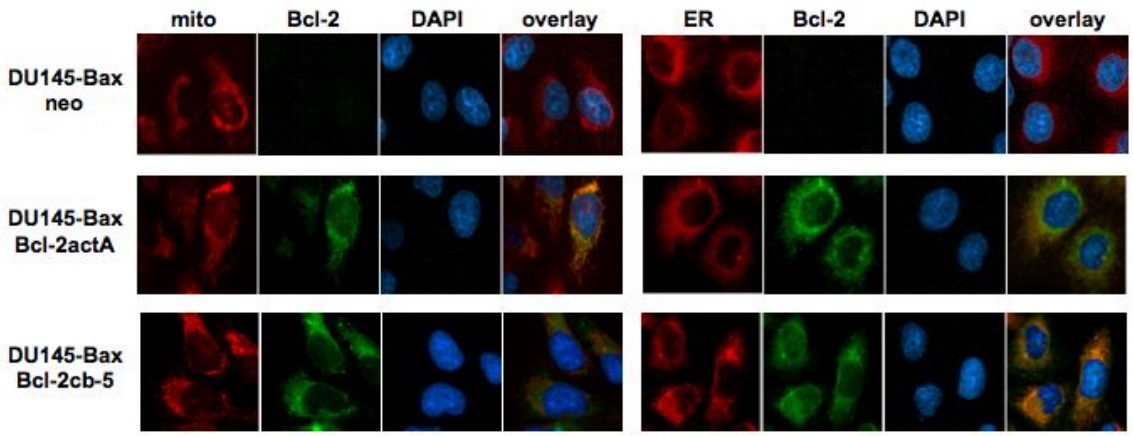

Figure 18: Localization of targeted Bcl-2 in DU145-Bax transfectants

Cells were stained for mitochondria with MitoTracker Red CMXRos or ER with mouse anti-BAP31 followed by Alexa Fluor 594-conjugated chicken anti-rat (red fluorescence). Bcl-2 was visualized by anti-Bcl-2 followed by Alexa Fluor 488-conjugated chicken anti-mouse (green fluorescence). The nuclei were stained in blue with DAPI.

The subsequent drugs, known for their involvement in cell death pathways and stimulation of different types of cell death were applied to confirm the functionality of cell system used in the following experiments. Epirubicin is a DNA damaging agent that inhibits topoisomerase II and triggers the mitochondrial apoptotic pathway. Tunicamycin, a protein N-glycosylation inhibitor, and thapsigargin, which blocks SERCA, are both causing ER stress induced apoptosis. After 72h, the cells were subjected to flow cytometric measurement of the cellular DNA content to determine the rate of apoptotic cells (figure 19). DU145-Bax neo cells showed 77% apoptotic cells after treatment with Epirubicin. Induction of ER stress also led to apoptosis in these cells. 74% of dead cells were detected after tunicamycin and 37% after thapsigargin treatment and showed partial inhibition of apoptosis as compared to the control transfectants. Epirubicin induced in 45% of DU145-Bax Bcl-2actA (mito) cells apoptosis by DNA damage. Treatment of DU145-Bax Bcl-2actA cells with tunicamycin and thapsigargin respectively induced in 52% and 47% of the cells apoptosis by ER stress. The highest inhibition of apoptosis by Bcl-2 was achieved in DU145-Bax Bcl-2cb5 (ER) cells. Whereas 50% of the cells died upon Epirubicin treatment, tunicamycin and thapsigargin had a minor effect on the cells by revealing only 25% and 22% of apoptotic cells. Thus, ER stress inducing agents induced much less apoptosis in cells that express Bcl-2 targeted to the endoplasmic reticulum. Additionally, these measurements suggested that Epirubicin does not engage to the ER to induce apoptosis.

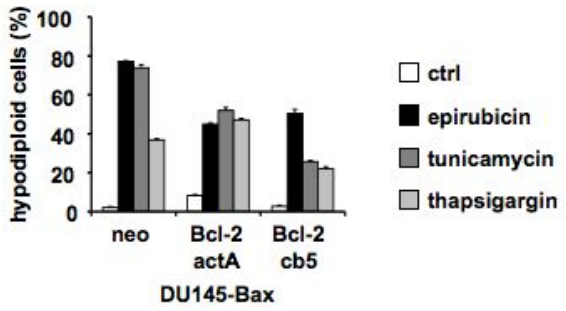

Figure 19: Functionality of the DU145-Bax Bcl-2 transfectants

DU145-Bax mock and Bcl-2 transfectants were treated with epirubicin (1µg/ml), tunicamycin (1µM), or thapsigargin (10µM). Apoptotic cells were determined after 72h by measurement of the genomic DNA content.

4.6.2 Inhibition of Bim induced apoptosis by Bcl-2

All three DU145-Bax cell lines were transduced with $AdBim_L$ and $AdBim_S$ respectively (figure 20). 48h post transduction, the apoptotic cells were measured by flow cytometry. Non-treated cells and cells under off conditions did not show any pertinent apoptosis neither for Bim_L nor for Bim_S. In DU145-Bax neo cells $AdBim_L$ induced 50% of cell death, whereas $AdBim_S$ managed to kill 63% of the cells. But when Bcl-2 was present, this anti-apoptotic protein was able to overcome the induction of apoptosis by Bim. Localized at the mitochondria Bcl-2 partially protected the cells against Bim. DU145-Bax Bcl-2actA cells died by 31% over Bim_L and by 45% over Bim_S expression. In consideration of the DU145-Bax neo cells, this signifies an inhibition of cell death by approximately 1.5 fold for both Bim_L and Bim_S. The most significant effect of Bcl-2 was detected in the DU145-Bax Bcl-2cb5 cells. The apoptotic rate was only 17% for Bim_L and 15% for Bim_S. Compared to the control cells this means an inhibition factor of 3 times for $AdBim_L$ and even 4 times for $AdBim_S$. Although Bim induced apoptosis in cells without Bcl-2 expression, as could be observed in the DU145-Bax neo cells, Bcl-2 protected the cells from death, especially when located at the endoplasmic reticulum. In this experiment as well as in the following it should be noted that Bim_S induced a higher level of cell death as has already be seen in the other DU145 cells. Once again the results confirm the current model that Bim_S induces apoptosis more effectively than Bim_L.

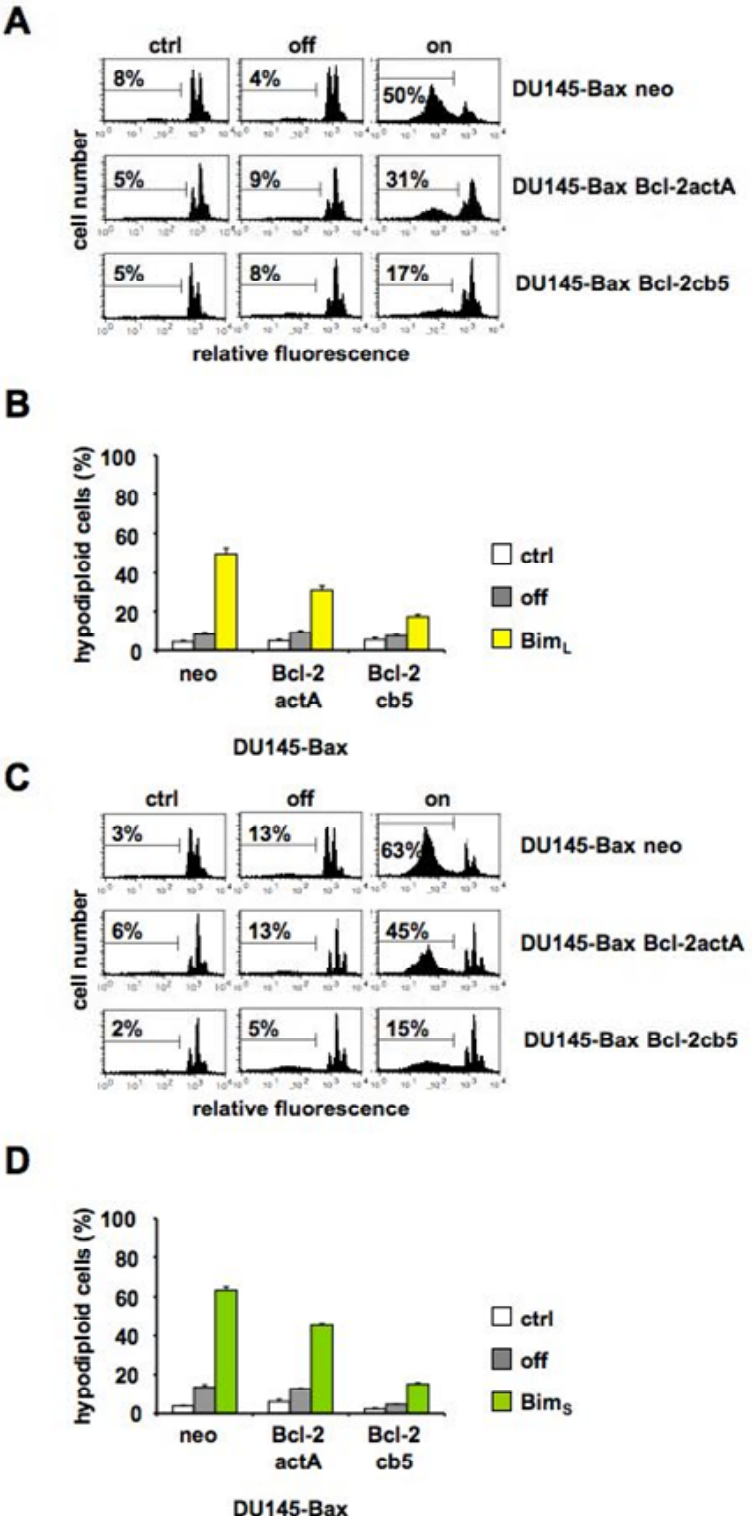

Figure 20: Bcl-2 at the endoplasmic reticulum protects cells from Bim-induced apoptosis

B, D: *flow cytometric detection of apoptotic cells based on measurement of the cellular DNA content. DU145-Bax cells were transduced with AdBim$_L$ or AdBim$_S$ and cultured in the presence (off) and the absence (on) of doxycyclin. Control cells were mock treated and grown in the absence of doxycyclin for 48h. Means +/- SD from three independent experiments.*
A, C: *representative histograms of experiments in* ***B*** *and* ***D****.*

4.6.3 Necrotic cell death

Since there were almost no apoptotic cells found among DU145-Bax Bcl-2cb5 cells upon Bim expression, the possibility of a necrotic cell death had to be ruled out. By double staining of the cells with Annexin-V-FITC and propidium iodide (PI), viability of the cells can be monitored and necrotic versus apoptotic cells identified. PI is used as a marker for the integrity of the cell membrane in PI negative cells. Annexin-V-FITC points at early apoptotic cells by binding to phosphatidylserine on the outer side of the membrane. It should be noted that in late stages of apoptosis cells may also lose their cell membrane integrity and can therefore be double stained. In that case, late apoptotic cells cannot be distinguished from necrotic cells. DU14- Bax neo, DU145-Bax Bcl-2actA and DU145-Bax Bcl-2cb5 cells were harvested 16h after transduction and were evaluated for double positive cells (figure 21). Non-treated cells that were used as a control, showed 5% necrosis in DU145-Bax neo, 8% in DU145-Bax Bcl-2actA and 3% in DU145-Bax Bcl-2cb5 cells. Culture of adenovirally transduced cells in the presence of doxycyclin to inhibit induction of Bim_L or resulted in a similarly low amount of necrotic cells. 5-6% was determined in DU145-Bax neo, 8-9% in DU145-Bax Bcl-2actA and 4-5% in DU145-Bax Bcl-2cb5 cells. Expression of Bim_L led to elevated, but not high levels of double positive cells. In DU145-Bax neo 21% of double positive cells were measured, in DU145-Bax Bcl-2actA 14% and in 16% DU145-Bax Bcl-2cb5 cells. The short variant of Bim did not particularly change the number of cells stained for Annexin-V-FITC and PI. Quantification of DU145-Bax neo, DU145-Bax Bcl-2actA and DU145-Bax Bcl-2cb5 cells revealed 24%, 20% and 21% of necrotic cells upon Bim expression.

These data clarified that Bcl-2actA or cb5 expression did not convert the cell death from apoptosis to necrosis but maintained viability upon Bim_L or Bim_S expression. The most relevant result though, was that in DU145-Bax Bcl-2cb5 cells Bim did not induce necrosis, nor did it cause apoptosis as could be observed by measurement of DNA-fragmentation (figure 20). Bcl-2 localized at the endoplasmic reticulum seems to prevent apoptotic death induced by Bim.

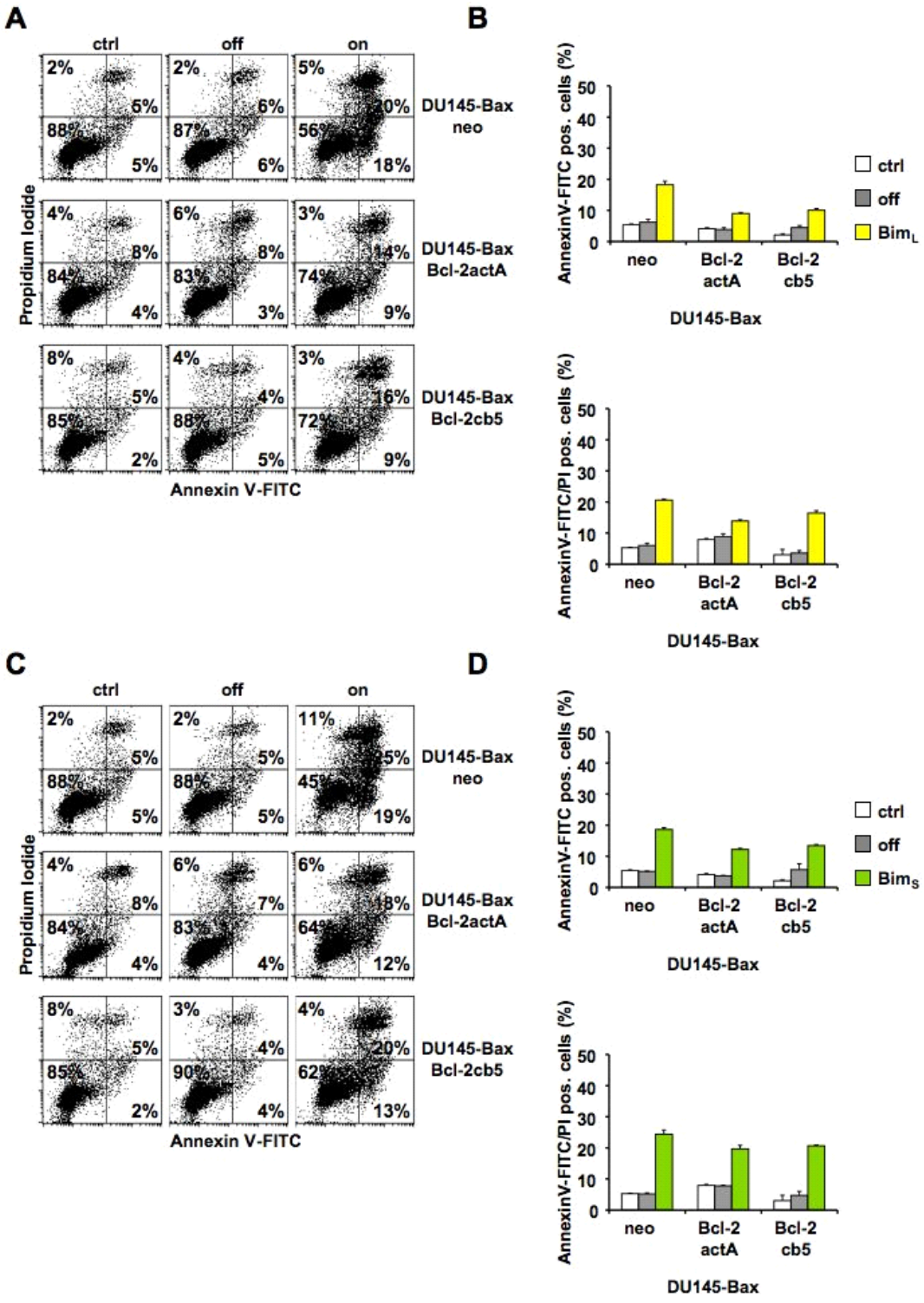

Figure 21: Bim does not induce necrosis in DU145 cells

A, C: *representative dot plots of experiments presented in* ***B, D****.*
B, D: *Cells were stained with Annexin-V-FITC, counterstained with PI and measured by flow cytometry. PI positivity is a sign of necrosis, whereas cells positive for Annexin-V, but negative for PI are defined as apoptotic. DU145 cells were transduced with AdBim$_L$ or AdBim$_S$ and cultured in the presence (off) and the absence (on) of doxycyclin. Control cells were mock treated and grown in the absence of doxycyclin for 48h. Means +/- SD from three independent experiments.*

4.7 Disruption of the mitochondrial membrane potential

The breakdown of mitochondrial membrane potential occurs prior to DNA-fragmentation and cell death and marks the activation of the intrinsic mitochondrial apoptotic pathway. Permeabilization of the mitochondrial outer membrane results in breakdown of the mitochondrial membrane potential. The next question was whether Bcl-2 was able to avoid cell death by preventing the breakdown of the mitochondrial membrane potential. DU145-Bax neo, DU145-Bax Bcl-2actA and DU145-Bax Bcl-2cb5 cells were incubated with $AdBim_L$ or $AdBim_S$ for 48h and the loss of the mitochondrial membrane potential was measured by flow cytometry.

Control treated cells of all three cell transfectants (neo, Bcl-2actA, Bcl-2cb5) did not show loss of the mitochondrial potential in the cells measured. This was still true when the expression of Bim was prevented under off conditions in AdBim transduced cells (figure 22). Under on conditions on the other hand, 62% of DU145-Bax neo cells were detected with mitochondrial permeability shift upon Bim_L and 79% upon Bim_S expression. Overexpression of Bcl-2 at the mitochondria reduced the cells with low mitochondrial membrane potential after Bim expression. Only 28% of D145-Bax Bcl-2actA cells were found to be affected by $AdBim_L$, whereas 35% of the cells showed loss of mitochondrial membrane potential in the case of $AdBim_S$. This indicated that these cells are protected by Bcl-2 at the mitochondrial level from Bim induced breakdown of the mitochondrial membrane potential. Conversely, in DU145-Bax Bcl-2cb5 cells, where Bcl-2 is associated to the endoplasmic reticulum, 54% of the cells had loss of their mitochondrial potential upon $AdBim_L$ infection. $AdBim_S$ caused in 67% of the same cells reduction of the mitochondrial potential. Thus, Bcl-2 could only to a minor extent prevent disruption of the mitochondria upon Bim expression, when it was targeted to the ER, although it dramatically inhibited DNA-fragmentation at the same position.

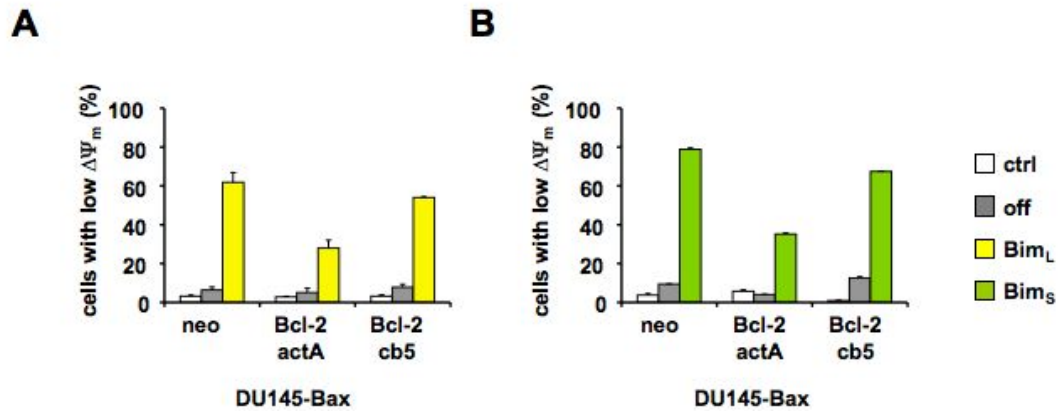

Figure 22: Bcl-2 targeted to the mitochondria prevents loss of $\Delta\Psi_m$ induced by Bim

A, B: *Flow cytometric detection of cells with lowered mitochondrial membrane potential. Indicated DU145 cells were transduced with $AdBim_L$ or $AdBim_S$ and cultured in the presence (off) and the absence (on) of doxycyclin. Control cells were mock treated and grown in the absence of doxycyclin for 48h. Means +/- SD from three independent experiments.*

4.8 Caspases are crucial in the Bim pathway

A connection between Bim and the caspases was already established in Bax or Bak expressing cells. Overexpressed Bcl-2 had been shown to save cells from apoptosis. Therefore, the question was addressed whether Bcl-2 would have an influence on the initiator caspases-8 and -9, and the effector caspase-3, which can be cleaved and activated by both initiator caspases. DU145-Bax neo, DU145-Bax Bcl-2actA and DU145-Bax Bcl-2cb5 cells were transduced with $AdBim_L$ or $AdBim_S$ and grown under off or on conditions. Cells were measured after 36h by flow cytometry for activated caspases using a cell permeable FITC labelled tetrapeptide of the corresponding caspase. The caspase activity was quantified according to elevated fluorescence. First, caspase-9 was analyzed in the Bim-induced apoptosis pathway. All three DU145-Bax transfectants, showed no caspase-9 activation upon mock treatment as determined by binding of the LEHD-FITC substrate (figure 23). Also, none of the cells showed caspase-9 activation when the expression of either Bim isoforms was suppressed by the presence of doxycyclin. Transduction of Bim_L in DU145-Bax neo resulted in 23% of cells with caspase-9 activity. In DU145-Bax Bcl-2actA transfectants 14% of cells were found with caspase-9 activation and in 8% of the cells with Bcl-2 localized at the ER. The same procedure upon Bim_S expression revealed caspase-9 activation in 44% of the DU145-Bax neo cells, 25% of the DU145-Bax Bcl-2actA transfectants and 16% of the DU145-Bax Bcl-2cb5 transfectants.

As the effector caspase-3 is cleaved and activated by caspase-9, caspase-3 activation should occur upon Bim expression. Cells, which were mock treated or transduced with $AdBim_L$ and then grown under off conditions, did not display caspase-3 activation as

determined by binding of fluorescin conjugated DEVD peptide. 39% of DU145-Bax neo cells presented caspase-3 activation upon Bim_L expression. $AdBim_S$ induced caspase-3 activation in 62% of the DU145-Bax neo cells. In cells, where Bcl-2 was targeted to the mitochondria, Bim_L and Bim_S expression stimulated in 17% and 37% of the cells caspase-3 activation, respectively. Similar numbers were obtained for DU145-Bax Bcl-2cb5 cells; upon Bim_L expression 22% and upon Bim_S expression 34% of the cells were determined.

When caspase-3 is active, it can process caspase-8, which in turn cleaves Bid to tBid. Truncated Bid (tBid) can provoke activation of the mitochondria via Bak and Bax, thereby creating an amplification loop. To see whether Bim could induce such a feedback loop, the activation of caspase-8 was measured by staining with a fluorescin labelled IETD peptide. Control treated cells of all three DU145-Bax transfectants were monitored for caspase-8 activation and showed no induction of such activation. Likewise, adenovirally transduced cells cultured in the presence of doxycyclin did not show caspase-8 activation. Transduction of DU145-Bax neo cells with $AdBim_L$ resulted in 30% of cells showing caspase-8 activation as compared to 14% of DU145-Bax Bcl-2actA cells and 15% of DU145-Bax Bcl-2cb5 cells were found. Bim_S had a much stronger effect on caspase-8 activation as compared to Bim_L. 64% of the DU145-Bax neo cells showed caspase-8 activation. But when Bcl-2 was present at the mitochondria caspase activation found in only 36% of the cells and when Bcl-2 was expressed at the ER, 25% of the cells showed caspase-8 activation.

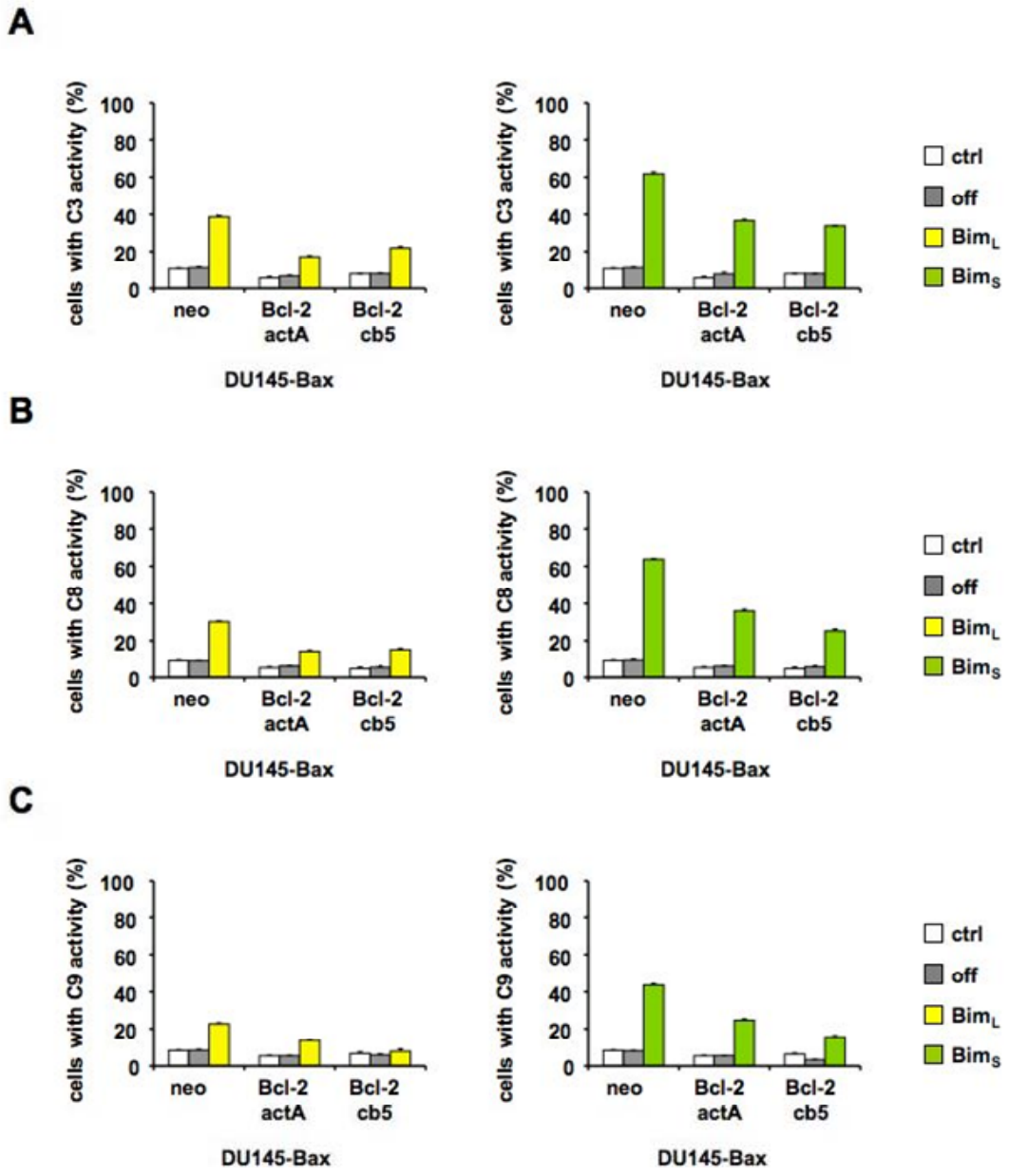

Figure 23: Bcl-2 targeted to the ER can prevent activation of the caspases upon Bim expression

A, B, C: *Flow cytometric detection of cells with increased caspase activation. Indicated DU145 cells were transduced with AdBim$_L$ or AdBim$_S$ and cultured in the presence (off) and the absence (on) of doxycyclin. Control cells were mock treated and grown in the absence of doxycyclin. Measurements were performed after 36h. Means +/- SD from three independent experiments.*

As observed in previous experiments Bim$_S$ exerted caspase activation in a higher percentage of cells (figure 23). Caspase activation for all caspases analyzed could be inhibited by both Bcl-actA and Bcl-2cb5 to a similar extent. To summarize these findings, Bim$_L$ and Bim$_S$ induced activation of caspase-3, -8, and -9, cells were infected with adenoviral Bim and treated with several irreversible caspase inhibitors. 48h later the cells were harvested and validated for DNA-fragmentation. Control treated DU145-Bax neo

cells displayed apoptosis in 3 to 4% of the cells (figure 24). Mock treated DU145-Bax Bcl-2actA cells showed 4-6% of apoptotic cells and DU145-Bax Bcl-2cb5 cells 2-6%. When these cells were infected with $AdBim_L$ or $AdBim_S$, but cultured in doxycyclin containing media, 5-7% DU145-Bax neo cells were found to be apoptotic, in DU145-Bax Bcl-2actA 4-8%, and in DU145-Bax Bcl-2cb5 5-11% of the cells. When the expression of Bim_L was turned on DU145-Bax neo cells genomic DNA fragmentation was measured in 52% of the cells. Additional treatment with zDEVD-fmk, a caspase-3 inhibitor, diminished the percentage of apoptotic cells to 25% establishing the relevance of the effector caspase-3 in this setting. When zLEHD-fmk was used to inhibit caspase-9, DNA fragmentation was measured in 44% of the cells. Caspase-8 inhibition by the zIETD-fmk inhibitor revealed an apoptotic rate of 31%. Caspase-4 has been shown to be located at the ER and was suggested to be only involved in apoptosis if the ER pathway is activated. Treatment with the caspase-4 inhibitor, zLEVD-fmk, marginally inhibited apoptosis with only 40% of cells showing DNA fragmentation. Finally, treatment with a pan-caspase inhibitor, zVAD-fmk, strongly reduced cell death to 10%, indicating that Bim_L cannot mediate apoptosis without caspases.

Even though apoptosis was decreased in cells expressing Bcl-2actA compared to cells with neo control transfectants showing only low Bcl-2 expression, additional caspase inhibition could further decrease apoptosis. DU145-Bax Bcl-2actA cells presented an apoptotic rate of 31% upon Bim_L expression, which fell to 22%, when the cells were additionally treated with zDEVD-fmk for caspase-3 inhibition. Bim_L triggered apoptosis in 27% of the cells in the presence of the caspase-9 inhibitor zLEHD-fmk. When caspase-8 was inhibited 14% of the DU145-Bax Bcl-2actA cells revealed DNA-fragmentation and inhibition of caspase-4 resulted in 23% of dead cells. Addition of the pan-caspase inhibitor zVAD-fmk decreased the rate of the apoptotic DU145-Bax Bcl-2actA cells to a level compared to the control treated cells, namely 7%. None of the caspase inhibitors had a major effect on cells overexpressing ER-targeted Bcl-2, since Bim_L alone could not induce cell death in more than just 16% of the cells. Addition of the caspase-3 inhibitor zDEVD-fmk led to 13% of cell death in these cells transduced with $AdBim_L$ and upon blocking of caspase-9 by zLEHD-fmk to 15%. Inhibition of caspase-8 by zIETD-fmk led to DNA-fragmentation in 9% of the cells and addition of the caspase-4 inhibitor zLEVD-fmk resulted in apoptotic DNA fragmentation in 13% of the cells. At last, when the cells were treated with the broad spectrum caspase inhibitor zVAD-fmk, 7% of apoptotic cells were measured.

The same settings were used for investigations of caspases involved in Bim_S mediated apoptosis. Bim_S caused apoptosis in 69% of DU145-Bax neo cells. Once caspase-3 was block by the inhibitor, the apoptotic rate was lowered to 44%. Caspase-9 inhibition still

resulted in 62% of apoptotic cells. When the casapse-8 inhibitor was added, Bim_S induced DNA fragmentation in 46% of the cells. Treatment with the casapse-4 inhibitor zLEVD-fmk decreased apoptosis to 48%. The pan-caspase inhibitor zVAD-fmk strongly reduced cell death to 15% of the cells. DU145-Bax Bcl-2actA cells displayed in 50% of the cells apoptotic DNA fragmentation upon Bim_S expression, showing that Bcl-2 was partially able to guard the cells. When casapse-3 was inhibited with zDEVD-fmk, 27% of apoptotic cells were observed. In case of additional caspase-9 inhibition, 45% of these cells underwent apoptosis. 26% of apoptosis happened in cells exposed to zIETD-fmk to block caspase-8 and 34% of cells died upon additional caspase-4 inhibition. In the presence of the pan-caspase inhibitor zVAD-fmk 20% of these cells showed DNA-fragmentation. Protection by Bcl-2cb5 was so strong that only 15% of apoptotic cells were detected in $AdBim_S$ infected cells. There was no relevant reduction in the amounts of apoptotic cells after additional treatment with the various caspase inhibitors. 11% of the cells were quantified for DNA-fragmentation upon Bim_S expression after addition of the caspase-3 inhibitor.14% of the cells underwent apoptosis when they were treated with casapse-9 inhibitor. Inhibition of caspase-8 and caspase-4 resulted in 12% and 13% apoptotic cells, respectively. The 12% of apoptotic cells detected in cells treated with the broad spectrum caspase inhibitor zVAD-fmk showed that the residual apoptosis induced by Bim_S in Bcl-2cb5 expressing cells is caspase independent.

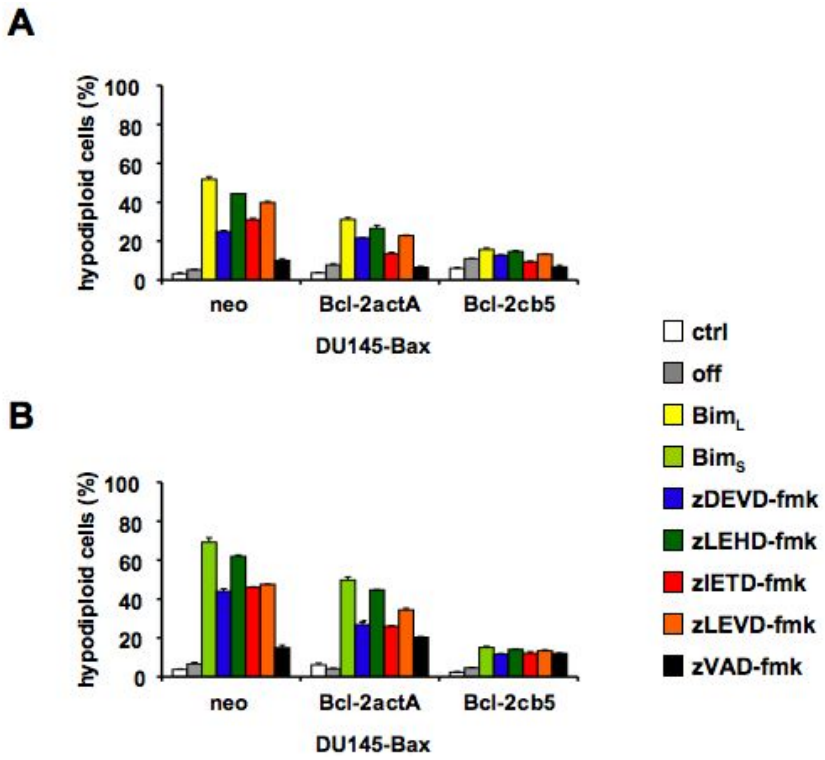

Figure 24: Bim acts in a caspase-dependent manner

***A**: DU145-Bax cells were infected with AdBim$_L$ in the presence (off) and the absence (on) of doxycyclin and treated with 20µM of indicated caspase inhibitors. Cells were cultured for 48h and measured for hypodiploid DNA content by flow cytometry. Means +/- SD from three independent experiments.*
***B**: DU145-Bax cells infected with AdBim$_S$ and treated as in **A**. Means +/- SD from three independent experiments.*

4.8.1 Processing of caspases upon Bim expression

Caspases were shown to be functionally involved in Bim induced apoptosis. This was shown on one hand by measurement of active caspases and on the other hand by the impact of inhibition of these caspases on apoptosis execution as measured by DNA fragmentation. Thus, detection of caspase subunits by Western blot analysis was used to show proteolytic processing of the caspase proforms (figure 25). All three DU145-Bax transfectants were transduced with the adenoviral vectors and harvested after 30h. The first lane demonstrates that, under on conditions, both Bim isoforms were expressed at equal levels in all cell lines. It should be noted that cells, which were infected with the adenovirus but kept under off conditions did not reveal Bim expression indicating tight conditional expression. Since expression of Bim in DU145-Bax neo cells induced loss of the mitochondrial membrane potential, cytochrome *c* release was analyzed. Cells, which were either control treated or grown under off conditions following adenoviral transduction, did not show cytochrome *c* release. In both cases, total cytochrome *c* was found in the mitochondrial fraction, regardless of the cell types and the Bim isoform used. In the

cytosolic fraction of DU145-Bax neo cells, cytochrome *c* could be detected upon both Bim_L and Bim_S indicating cytochrome *c* release expression. Expression of Bcl-2 at the mitochondria mostly prevented release of cytochrome *c* into the cytosol upon Bim_L expression. The same result could be found upon Bim_S expression where a subtle band for cytochrome *c* was seen in the cytosolic fraction. Surprisingly, expression of Bcl-2cb5 at the ER inhibited mitochondria from releasing cytochrome *c* in the presence of Bim_L and Bim_S, respectively. This indicates a crosstalk of the ER pathway feeding into mitochondrial apoptosis signalling. Downstream of cytochrome *c* release formation of the apoptosome and the activation of caspase-9 occur. Uncovering of caspase-9 subunits would indicate processing of caspase-3 by the mitochondrial pathway although caspase-3 was also shown to cleave the caspase-9 zymogen. Bim_L expression in DU145-Bax neo cells led to processing of caspase-9 as could be observed by the reduced proform. In the same cells, Bim_S also induced cleavage of caspase-9. Detection of decreased pro-caspase-9 in DU145-Bax Bcl-2actA cells upon expression of Bim_L showed that caspase-9 was processed even in the presence of Bcl-2. Transduction of these cells with $AdBim_S$ under on conditions led to the detection of the processed caspase-9 subunit, indicating activation of caspase-9. In DU145-Bax Bcl-2cb5 cells, neither expression of Bim_L nor Bim_S caused cleavage of caspase-9. No reduction of the proform and no cleavage product of caspase-9 could be detected upon Bim expression. Bcl-2 localized at the ER might interfere with Bim mediated caspase-9 activation in a yet unknown way. According to casapse-9 processing, caspase-3 cleavage product was seen upon Bim_L expression in Bcl-2 deficient cells. Processing of caspase-3 proform was found in DU145-Bax neo cells upon Bim_S expression. DU145-Bax Bcl-2actA and DU145-Bax Bcl-2cb5 cells revealed a slight occurrence of caspase-3 p20 fragment in case of Bim_L or Bim_S expression. To enhance the death signal, caspase-3 can process and activate caspase-8 creating a positive feedback loop. No matter whether Bcl-2 was overexpressed in the cells or not, expression of Bim_L or Bim_S resulted in the appearance of a processed caspase-8 intermediate. This might indicate that the amplification loop was initiated independently of the Bcl-2 status or that caspase-8 processing is initiated upstream of a Bcl-2 inhibitable event. Finally, cleavage of PARP was examined as it is cleaved by caspase-3 upon apoptotic stimuli. DU145-Bax neo cells transduced with $AdBim_L$ revealed PARP cleavage under on conditions. In the same settings, upon expression of Bim_S only the cleavage product of PARP could be detected indicating massive caspase-3 activation. Despite the expression of Bcl-2actA, Bim_L as well as Bim_S expression induced PARP cleavage to an extent comparable to the DU134-Bax neo controls. Furthermore, Bim_L and Bim_S triggered PARP cleavage under on conditions in DU145-Bax Bcl-2cb5 cells, although to a lesser extent. This indicates that caspase activation leading to PARP processing via caspase-3

like caspases occurs within the ER pathway downstream of Bcl-2 inhibitable events.

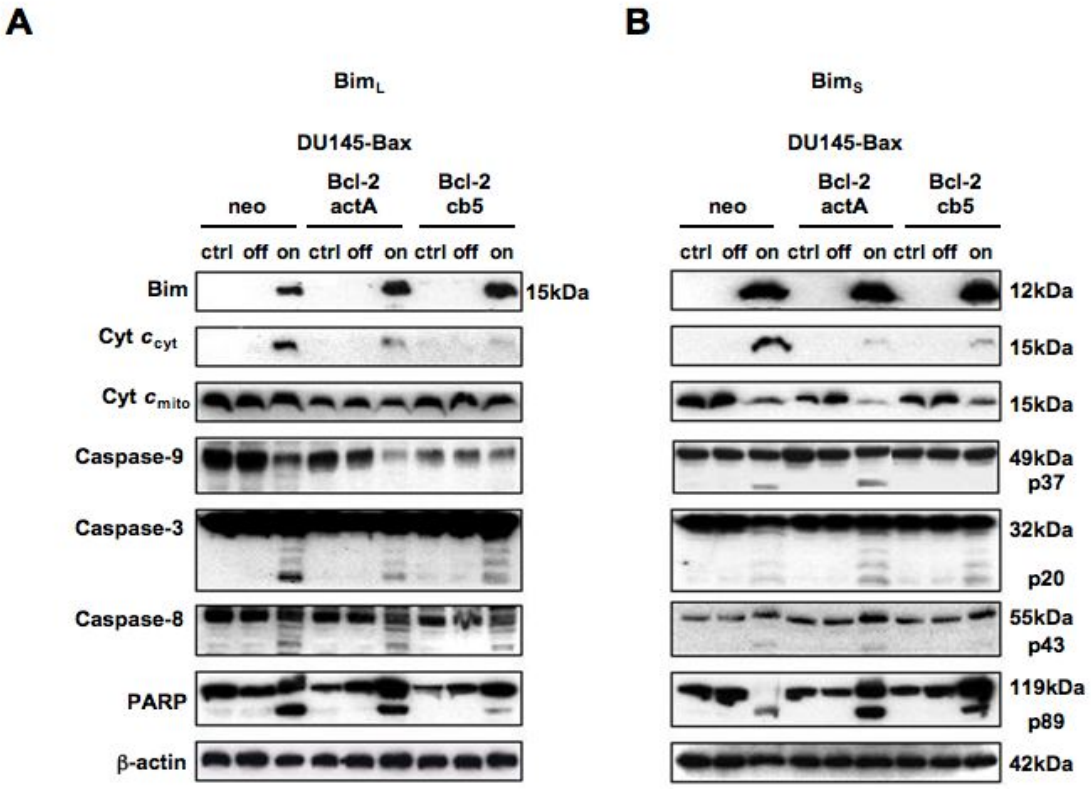

Figure 25: Bcl-2 partially suppresses processing of caspases

A: *Western blot analysis for indicated pro-apoptotic proteins. DU145-Bax cells were transduced with AdBim$_L$ and cultured for 30h in the presence (off) and the absence (on) of doxycyclin. Cell lysates were blotted for the indicated proteins with the appropriate antibodies.*
B: *DU145-Bax cells were transduced with AdBim$_S$, and treated as in **A**.*

4.9 Caspase-8 is involved in Bim$_S$ induced apoptosis

Although the extrinsic death-receptor initiated pathway was not investigated with regard to Bim mediated apoptosis, it is possible that caspase-8 is important in the Bim pathway since it was reported that caspase-8 is involved in ER stress induced apoptosis (Chandra, et al., 2004).

DU145-Bax neo, DU145-Bax Bcl-2actA and DU145-Bax Bcl-2cb5 cells were incubated with AdBim$_S$ for 24h and cells with low mitochondrial membrane potential were measured by flow cytometry (figure 26). DU145-Bax neo cells mock treated or grown under off conditions did not show cells with low mitochondrial membrane potential. Expression of Bim$_S$ led to 60% of cells, which had loss of their mitochondrial potential and 58% were detected when these cells were treated with casapse-8 inhibitor. DU145-Bax Bcl-2actA cells, which were either not infected with the adenovirus AdBim$_S$ or were infected, but

cultured in the presence of doxycyclin showed 4-6% of cells with low mitochondrial membrane potential. 46% of these cells were detected with mitochondrial permeability shift upon Bim_S expression. Additional inhibition of caspase-8 reduced the effect by half. Similar data was obtained in cells, where Bcl-2 is targeted to the ER. Here, 7% of cells showed mitochondrial membrane potential loss when control cultured or transduced with $AdBim_S$ in the presence of doxycyclin. Bim_S induced breakdown of the mitochondrial membrane potential in 58% of the cells. This number was reduced to 32% when caspase-8 was blocked. Thus, Bim_S mediates cell death through caspase-8 in a Bcl-2 dependent way. According to these data, no difference could be detected with respect to caspase-8 inhibition in Bim_S induced mitochondrial permeabilization whether Bcl-2 was expressed at the mitochondria or at the ER. The fact that inhibition of caspase-8 did not influence the breakdown of the mitochondrial membrane potential might suggest that Bcl-2 is upstream of caspase-8 activation.

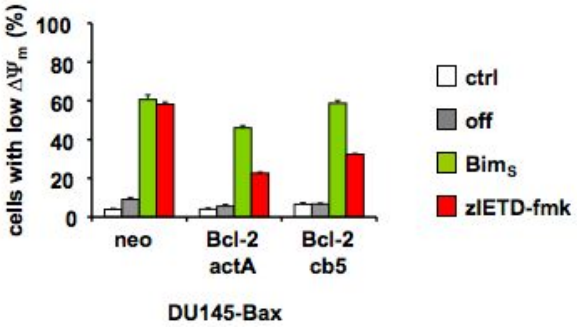

Figure 26: Bim_S mediates caspase-8 activation

DU145 cells were transduced with $AdBim_S$ and cultured in the presence (off) and the absence (on) of doxycyclin or were additionally treated with capase-8 inhibitor for 24h. Control cells were mock treated and grown in the absence of doxycyclin. Means +/- SD from three independent experiments.

4.10 Bim_S induces calcium release into the cytosol

The main calcium store in the cell is the endoplasmic reticulum. Ca^{2+} is pumped from the cytosol into the lumen of the endoplasmic reticulum by the SERCA (sarcoplasmic endoplasmic reticulum calcium ATPase). ER stress causes the depletion of calcium from the store. Cytosolic Ca^{2+} is mostly absorbed by the mitochondria through their Ca^{2+} uniporters. The positively charged calcium ions disturb the mitochondrial membrane potential. At a certain threshold, accumulated Ca^{2+} in the matrix triggers the opening of the permeability transition pore, which results in swelling of the matrix, the loss of the membrane potential and finally in the destruction of the outer mitochondrial membrane. Consequently, apoptosis inducing factors are released form the mitochondria, such as cytochrome *c*, Omi/Htr2 and Smac/Diablo. Since Bcl-2 can antagonize the apoptosis

promoting effect of Bim, it was investigated whether Bcl-2 is inhibiting apoptosis (see 3.5.2) by avoiding ER stress and interfering with Ca^{2+} release. Considering that a leak of calcium from the ER is one of the first events of the apoptotic process, even upstream of mitochondrial activation upon ER stress, Ca^{2+} fluxes were measured Bim expression. Cytosolic Ca^{2+} was measured by using Fluo-3AM, which binds to Ca^{2+} ions resulting in increased fluorescence. Time course experiment of Bim expression showed that Bim can be already found 8h after infection with the adenoviral vector and is increasing with time (figure 27). So, cytosolic Ca^{2+} elevations were determined at 8h, 16h and 24h by flow cytometry. As a positive control for ER tress, thapsigargin was used. This agent inhibits SERCA, and induces an unfolded protein response and increased Ca^{2+} levels in the cytosol. DU145-Bax neo, DU145-Bax Bcl-2actA or DU145-Bax Bcl-2cb5 cells were infected with either $AdBim_L$ or $AdBim_S$ or treated with thapsigargin and subjected to flow cytometric measurement (figure 27). Control cells with low Bcl-2, which were control treated or were transduced with $AdBim_L$ but grown under off conditions did not show any sign of calcium leak at any of the indicated time points. Turning on $AdBim_L$ expression did not result in major changes in these results. Even after 24h, only 10% of the cells were detected with elevated Ca^{2+} levels, implying that Bim_L cannot trigger Ca^{2+} release. The same cells were infected with $AdBim_S$ and displayed another picture. In DU145-Bax neo, mock treated and cells under off conditions did not show increased release of Ca^{2+} at any time points. But under on conditions, $AdBim_S$ provoked after 16h calcium fluxes in 19% of the cells. This number increased to 30% when the cells were exposed to $AdBim_S$ for 24h. Treatment of the cells with thapsigargin only, proved that ER stress could be induced in these cells. 8h after addition of thapsigargin, 15% of the cells were found to have released Ca^{2+}. 16h later, 36% and at 24h 44% of the cells were affected.

Overexpressed Bcl-2actA targeted to the mitochondria did not change the situation for the cells. Cells, which served as a negative control or in which the expression of either of the Bim isoforms was kept suppressed in the presence of doxycyclin, did not show Ca^{2+} fluxes. Expression of Bim_L did not have an impact on the cells in respect to Ca^{2+} release. Bim_S on the other hand, triggered Ca^{2+} release from the ER in DU145-Bax Bcl-2actA cells. 8h post infection nothing had happened yet, another 8h later though in 17% of these cells higher amounts of Ca^{2+} were measured. Cells, which were collected 24h after transduction with $AdBim_S$ showed 20% of cells with calcium release. Once these cells were treated with thapsigargin as a positive control, they showed high release rates of 42% at 8h, 55% at 16h and finally 58% at 24h. DU145-Bax Bcl-2actA cells seemed to be more sensitive for the ER stress agent. The fact, that these cells are transfectants might be the answer for this phenomenon. Finally, DU145-Bax Bcl-2cb5 cells were investigated. Since these cells express Bcl-2 at the ER, they should be protected against ER stress and

therefore should not release Ca^{2+}. Mock treated cells and cells, which were infected with either of the adenovirus but grown in the presence of doxycyclin, could not be monitored for Ca^{2+} fluxes. Indeed, neither of the Bim variants could induce Ca^{2+} release at any of the indicated time points under on conditions. Bcl-2 targeted to the ER was able to protect the cells from ER stress generated by Bim_S. Moreover, even thapsigargin was not strong enough to break through the protective wall of Bcl-2. After the longest incubation time of 24h with thapsigargin, 20% of the cells were detected with elevated Ca^{2+} levels, revealing the functionality of the cells. These results point to a link between Bim_S and Bcl-2 at the ER, which has yet to be identified.

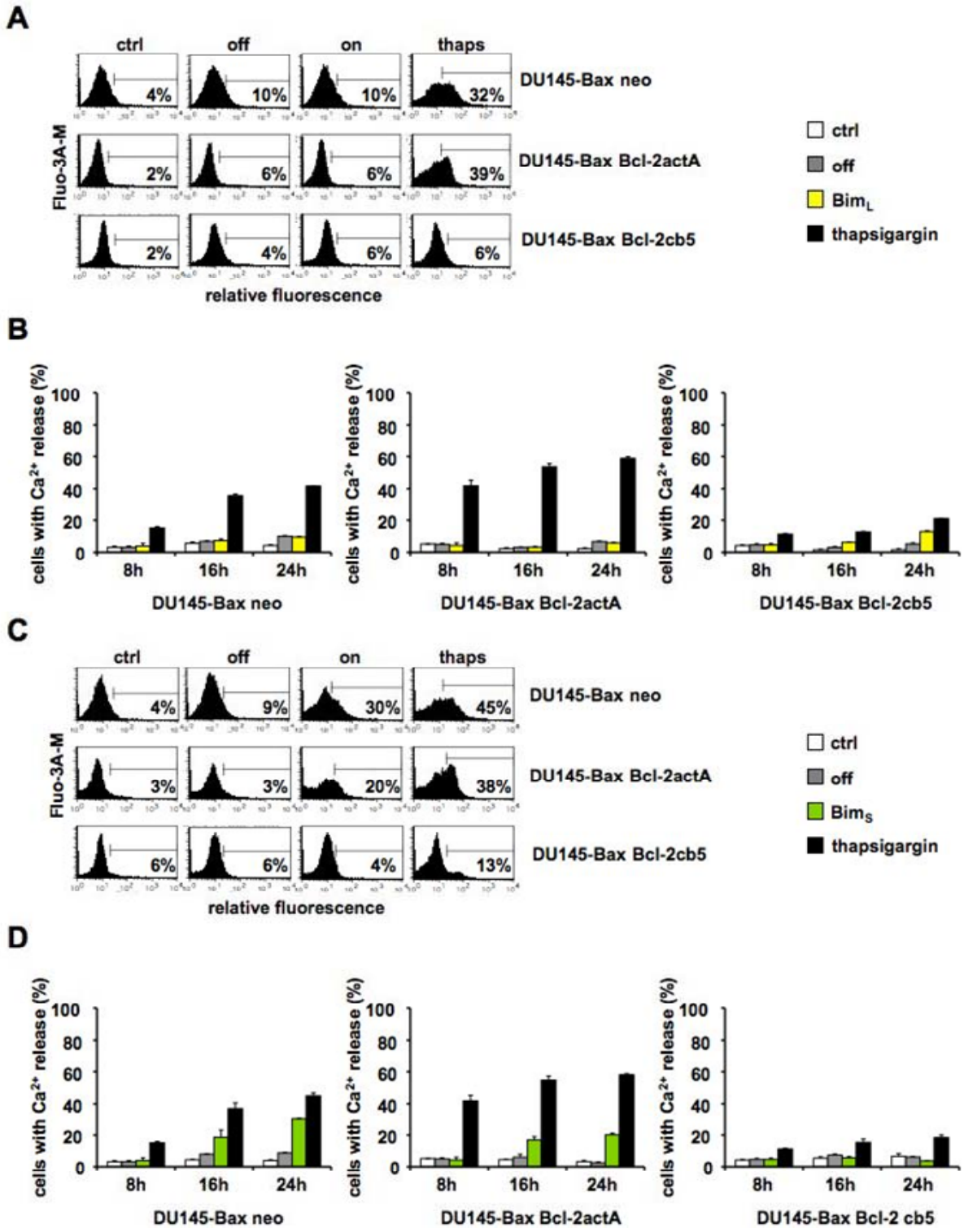

Figure 27: Ca^{2+}-fluxes from the ER upon Bim expression

***A, C**: representative histograms of Ca^{2+} release measurement after 24h.*
***B, D:** DU145-Bax cells were transduced with $AdBim_L$ or $AdBim_S$ and cultured with doxycyclin (off) or without doxycyclin (on) for indicated time points. As a positive control the cells were treated with 10µM thapsigargin (thaps). Increased cytosolic Ca^{2+} levels were measured by flow cytometry. Means +/- SD from three independent experiments.*

4.10.1 Bim induces upregulation of ER stress proteins

According to the results obtained so far, it was speculated that Bim might associate with the endoplasmic reticulum where it would induce a stress response and initiate apoptosis. Therefore, detection of ER protein levels associated with ER stress responses might give some insights into the mechanisms by which Bim mediates apoptosis. Total lysates were collected 16h post infection with either $AdBim_L$ or $AdBim_S$ and subjected to Western blot analysis (figure 28).

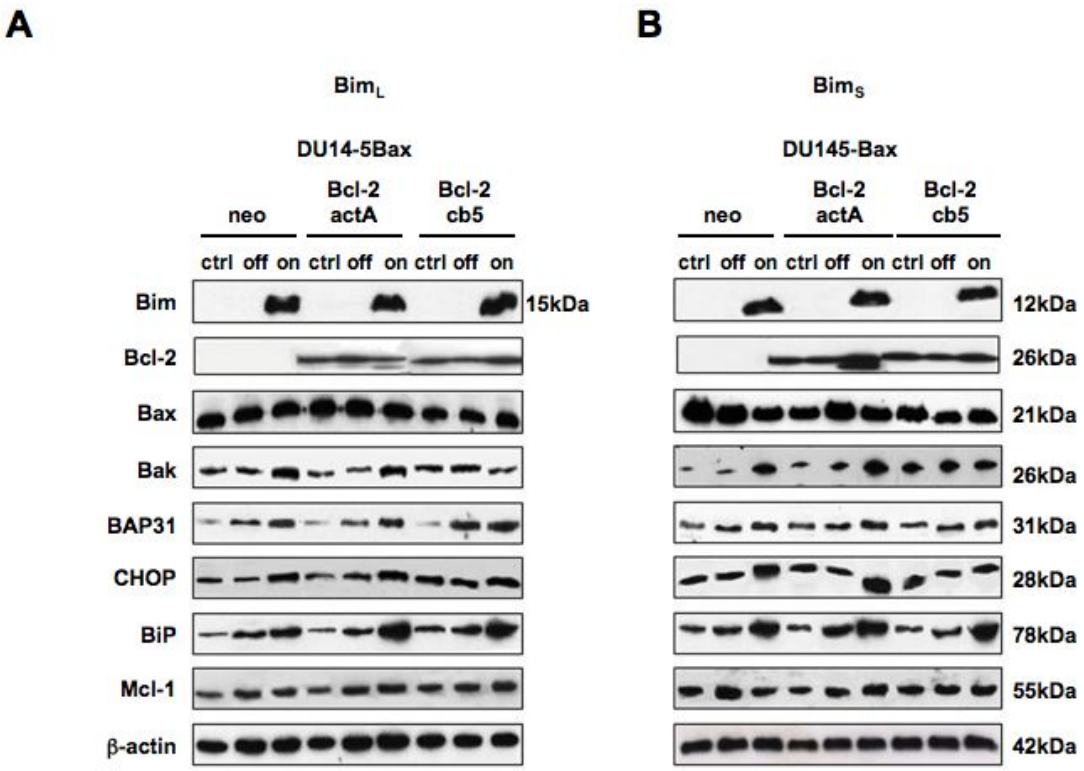

Figure 28: Bim induces upregulation of ER proteins

A: *Western blot analysis of the expression of ER associated proteins. DU145-Bax cells were transduced with $AdBim_L$ and cultured for 30h in the presence (off) and the absence (on) of doxycyclin. Cell lysates were blotted for the indicated proteins and developed with the appropriate antibodies.*
B: *DU145-Bax cells were transduced with $AdBim_S$, and treated as described in **A**.*

Detection of Bim_L (figure 28A) after transduction of $AdBim_L$ of all three cells lines confirmed that Bim_L was expressed under on conditions at comparable levels. None of the cell lines, which were mock treated or grown in the presence of doxycyclin, did show any Bim_L expression. Additionally, DU145-Bax neo, DU145-Bax Bcl-2actA and DU145-Bax Bcl-2cb5 were examined for Bcl-2 expression. Only cells that stably express Bcl-2, namely DU145-Bax Bcl-2actA and DU145-Bax Bcl-2cb5, revealed Bcl-2 expression. Since Bim activates Bax directly or indirectly expression levels might be changed upon Bim

expression, especially in cells, which express Bcl-2 and might neutralize Bim. But Detection of Bax revealed that it was equally expressed, no matter which cell type was examined. Moreover, cells under on conditions showed the same Bax level as cell under control or off conditions. For the same reason Bak expression was examined. $AdBim_L$ induced upregulation of Bak in DU145-Bax neo cells under on conditions. While Bim_L expression also resulted in upregulation of Bak in DU145 BaxBcl-2actA cells, Bak proteins levels remained the same when Bcl-2 was targeted to the ER. CHOP is a transcription factor induced during ER stress and promotes apoptosis. Bim_L expression triggered upregulation of CHOP in all three cell transfectants, indicating that Bim_L may have caused ER stress. The slightly elevated CHOP level detected under off conditions was most likely set off by the adenovirus. Another ER stress protein is BAP31which is a resident integral membrane protein of the endoplasmic reticulum. It regulates the export of other integral membrane proteins to the downstream secretory pathway. ER stress and other apoptotic stimuli lead to generation of a p20 fragment, preferably cleaved by caspase-8. Also BAP31 was found to be up regulated, but only in DU145-Bax neo and DU145-Bax Bcl-2actA cells, whereas in DU145-Bax Bcl-2cb5 cells no such upregulation could be determined upon Bim_L expression. BiP/GRP78, another ER stress protein, is involved in protein folding and assembly, targeting misfolded protein for degradation, ER Ca^{2+}-binding and controlling the activation of trans-membrane ER stress sensors. Further, due to its anti-apoptotic character, it is a component of the unfolded protein response. Upon Bim_L expression higher amounts of BiP were detected in all three cell transfectants. Cells, which were transduced with $AdBim_L$ in the presence of doxycyclin, presented a slightly stronger band than the corresponding control cells. This could be explained by a potential stress of infection with the adenovirus. The anti-apoptotic protein Mcl-1 is known to bind to Bim with high affinity, although its preferred binding partner is the BH3-only protein Noxa. Influence or binding of Bim to Mcl-1 cannot be ruled out, but nevertheless total Mcl-1 was equal in all three cell lines. Even expression of Bim_L did not influence Mcl-1 amounts. β-Actin detection was used as a loading control.

Detection of the same proteins was performed upon Bim_S expression. Transduction of $AdBim_S$ in all three transfectants under on conditions proved that $AdBim_S$ was equally expressed in all of them (figure 28B). Bcl-2 could not be found in DU145-Bax neo cells, but was present in the transfectants. Also the short version of Bim did not induce changes in the total amount of Bax expression. The cells expressed similar levels whether they were treated or not. Its multi-domain relative Bak on the other hand was upregulated in cells without Bcl-2 and also in cells, where Bcl-2 was localized at the mitochondria under on conditions. Control cells and cells, which were grown in the presence of doxycyclin showed unchanged Bak levels. In DU145-Bax Bcl-2cb5 cells expressing Bim_S no

difference could be determined. The protein band for Bak in Bim_S transduced cells in the absence of doxycyclin was alike to the ones observed under control conditions. Bim_S expression resulted in up regulation of CHOP in cells where Bcl-2 was absent. But Bcl-2 targeted to the mitochondria or the ER could not prevent induction of this ER protein under on conditions for Bim_S expression. Detection of BAP31 showed that up regulation occurs in DU145-Bax neo cells upon Bim_S expression and when Bcl-2 is localized at the mitochondria. Just as for Bim_L, no upregulation of BAP31 could be monitored in cells that express Bcl-2 at the ER. BiP displayed a higher expression in all three cells types upon Bim_L or Bim_S expression under on conditions, regardless of the Bcl-2 status. AdBim_S transduced cells showed a minor upregulation of the ER stress proteins CHOP and BiP under off conditions. Expression of Bim_S did not influence Mcl-1 on its expression level. All samples were quantified for equivalent expression. β-Actin detection served as a loading control for the Western blot analysis.

5 Discussion

5.1 Bim mediates apoptosis in Bax- or Bak-dependent manner and is antagonized by Bcl-2

The main function of Bax and Bak is to disrupt mitochondrial membrane integrity in order to release cytochrome *c* and other pro-apoptotic factors. It was proposed that they mediate this release either by binding to and modifying mitochondrial channel proteins such as VDAC or ANT (Marzo, et al., 1998) or by direct pore formation (Antonsson, et al., 1997; Eskes, et al., 1998). However, the activation of these two pro-apoptotic proteins is still controversial. Two different models have been discussed, the direct activation and the indirect activation. The direct activation model proposes that a subgroup of the BH3-only proteins called “activators” can activate Bak and Bax, whereas the remaining BH3-only proteins, called “sensitizers”, neutralize the anti-apoptotic proteins (Willis, et al., 2007). The indirect activation model suggests that Bak and Bax are held in check by being bound to anti-apoptotic proteins. BH3-only proteins displace Bak and Bax from the anti-apoptotic proteins to achieve their activation (Willis and Adams, 2005). But no interaction of Bak with BH3-only proteins could be detected. Also, Bax did not bind to Bim_{EL} and Bim_L in co-immunoprecipitation studies (Marani, et al., 2002; O'Connor, et al., 1998; Yamaguchi, et al., 2003). Even though Bim_S was found to weakly bind to Bax it might not be the physiological truth, since this Bim_S-Bax complex existed in detergents that alter Bax conformation. Additionally, a Bim_S mutant that was not able to bind to Bax anymore but could still interact with anti-apoptotic proteins and induce as much apoptosis as the wild type (Willis and Adams, 2005; Willis, et al., 2007). And finally, the ability of Bcl-2 or Bcl-x_L to inhibit apoptosis induced by Bid, Bim or Bad correlates with their ability to bind BH3-only proteins but not Bax and Bak (Cheng, et al., 2001). For these reasons it is more likely that the indirect activation of Bax and Bak by the BH3-only proteins is the main process.

Therefore, the investigations described in this study focused in the first part on clarification if Bax and Bak were redundant when Bim is overexpressed. With the help of an antibody specific for an epitope at the N-terminus of Bax or Bak the conformational change was examined. Conformational change of Bax and Bak became evident when elevated fluorescence was measured. Conformational change of Bax could be detected in DU145-Bax cells upon Bim_L and Bim_S expression, respectively (figure 11). According to the specific Bak antibody Bim_L as well as Bim_S induced Bak activation in DU145-Bak cells as confirmed by the measurement of the conformational change of Bak. Whereas monomeric Bax is translocated from the cytosol to the mitochondria, Bak already resides at the mitochondria. Nevertheless, both proteins undergo conformational change and oligomerize at the mitochondria, which seems to be based on the activation by Bim.

DU145 cells stably expressing EGFP-Bax or EGFP-Bak were used to detect clustering of these two pro-apoptotic proteins. Non-treated DU145 cells, which stably expressed either of both fusion proteins, indicated the localization of Bax and Bak. In DU145 EGFP-Bax cells the fluorescence of the fusion protein appeared diffuse being concordant with the cytosolic localization. EGFP-Bak on the other hand presented a string-like pattern, pointing to its association at the mitochondria (figure 9). When DU145 EGFP-Bax cells were transduced with adenoviral Bim_L or Bim_S clustering of Bax could be observed by the punctuated pattern. Expression of either of the two Bim isoforms in DU145 EGFP-Bak cells also led to clustering of the fusion protein, noticeable by the EGFP spots. These results show that Bim induces activation of pro-apoptotic the multi-domain proteins leading to conformational change and clustering of these proteins, which might represent oligomerization.

Although it is not possible to conclude direct or indirect activation of Bax and Bak from this study, it can be said that Bim induces Bax and Bak activation. Bax and/or Bak are needed for execution of apoptosis. Several investigations with *bax/bak* double knockout cells clarified that without these two proteins apoptosis will not occur (Armstrong, 2006; Lindsten, et al., 2000; Wei, et al., 2001). Expression of Bim_L resulted in apoptosis in DU145-Bax and DU145-Bak cells, but not in the DU145 mock cells, confirming that induction of apoptosis needs Bax or Bak. Bim_S joined in on this effect, by inducing cell death in cells expressing Bax or Bak, but not the control cells. Indeed, a higher rate of apoptosis was measured upon Bim_S expression. Activation of Bax and Bak upon Bim expression ultimately led to apoptosis determined by measurement of DNA-fragmentation. A high percentage of DU145-Bax cells were found to be apoptotic after transfection with $AdBim_L$ and $AdBim_S$, respectively. DU145-Bak cells showed similar rates of apoptosis upon Bim_L or Bim_S expression. These results demonstrate that Bim_L as well as Bim_S are able to kill in a Bax- and Bak-dependent manner.

After establishing the role of Bax and Bak in the Bim induced apoptotic pathway, the focus of this study turned to the relation of Bim to the mitochondria and the ER. The primary objective to use the DU145-Bax targeted Bcl-2 cell system was to assess whether Bim would activate the mitochondria or the ER to induce apoptosis. In transfectant cells Bcl-2 was targeted to the mitochondria by exchanging the C-terminal insertion sequence for an equivalent mitochondrial signal of (DU145-Bax Bcl-2actA) or to the ER by a transmembrane sequence (DU145-Bax Bcl-2cb5). DU145-Bax cells transfected with an empty vector served as a control (DU145-Bax neo). In the absence of Bcl-2 overexpression both Bim_L and Bim_S induced apoptosis in DU145-Bax neo cells, indicating their ability to activate Bax. However, in the presence of Bcl-2, Bim was at least partially blocked in its actions and therefore revealed its preferred pathway. Bcl-2 localized at the

mitochondria reduced the apoptotic rate upon Bim_L expression. A similar inhibitory effect of Bcl-2 was found for the apoptotic rate induced by Bim_S. Even though the mitochondria were protected by Bcl-2 from being permeabilized, Bim was able to induce cell death by other mechanisms. Overexpression of either Bim isoforms in DU145-Bax cells with ER localized Bcl-2 did not end in apoptosis. The anti-apoptotic function of Bcl-2 prevented Bim-induced activation of ER mediated apoptosis. These results implicate that Bim might aim to the ER on its pathway once it is activated by an apoptotic stimuli. This consideration is supported by the report that Bim translocated to the ER in murine myoblast cells (C2C12) upon tunicamycin treatment. There, Bim stimulates the activation of ER stress induced apoptosis, which could be prevented by overexpression of Bcl-x_L (Morishima, et al., 2004). Furthermore, Bim has been reported to respond to cell death downstream of ER stress and to be induced in response to ER stress. Additionally, it was shown that Bim targeted to the ER initiated apoptosis and can therefore act as a specific death signal in response to ER stress (Morishima, et al., 2004). Other studies reported that ER targeted Bcl-2 may sequester Bim, preventing it from interacting with other members of the Bcl-2 family (Egle, et al., 2004). This possibility was also stated by other reports, saying that Bim binds and inhibits ER localized Bcl-2 to regulate cell death and is therefore capable of regulating this anti-apoptotic protein at the ER (Kim, et al., 2004). It should be mentioned that Bcl-2 targeted exclusively to the ER is more restricted in its anti-apoptotic actions compared to Bcl-2 predominantly expressed at the mitochondria, suppressing cell death induced by ER stress agents and by c-Myc.

The secondary outcome of the overexpression of Bim in the DU145-Bax Bcl-2 system was the ability of membrane associated Bcl-2 to antagonize the killing effect of Bim. Whereas Bcl-2 targeted to the mitochondria only partially reduced Bim-induced apoptosis, localization of this anti-apoptotic protein at the ER almost completely cell death upon Bim expression. Similar results were found in other cell lines with different apoptotic stimuli. It was reported that Bcl-2 targeted to the mitochondria or the ER could protect cells from apoptosis depending on the cell death stimuli (Zhu, et al., 1996). Furthermore, it was also shown that Bcl-2 located at the ER was able to block cytochrome *c* release upon ER stress inducing agents (Hacki, et al., 2000).

5.2 Bim activates the mitochondrial apoptotic pathway

Mitochondria play a key role in executing the intrinsic pathway, but are also involved in the extrinsic pathway that leads to apoptosis in response to intracellular stress signals. There two types of cells; in cell type I the cell death signal is propagated by caspase cascade initiated by the activation of large amounts of capsase-8 at the DISC following followed by the rapid cleavage of caspase-3 which in turn cleaves vital substrates in the cell. In type II cells no DISC is formed; instead caspase-8 cleaves the pro-apoptotic protein Bid to tBid leading to activation of the mitochondrial cell death pathway (Krammer, 2000). The permeabilization of the outer mitochondrial membrane marks the initiation of the intrinsic pathway followed by formation of the apoptosome and the activation of the initiator caspase-9. In the extrinsic pathway the mitochondria are stimulated by the activation of caspase-8 and subsequent cleavage of Bid. Moreover, rupture of the mitochondria can be amplified through caspase-3, caspase-8 and the cleavage of Bid in a feedback loop. The main event of mitochondrial activation is the loss of the membrane potential and consequently the release of cytochrome *c* and different pro-apoptotic factors. Mitochondria appear to undergo several changes in membrane structure and morphology before releasing cytochrome *c*. All these changes appear to influence the release of cytochrome *c*. Several studies indicate that transient openings in the mitochondrial permeability transition pore are important for this event. Bcl-2 family proteins may activate several processes in the mitochondria and ER during apoptosis, for example the reorganization of proteins within the intermembrane space before their passage across the outer mitochondrial membrane (Breckenridge and Xue, 2004). There are several models for how cytochrome *c* is released from the mitochondria. Most of them concentrate on the mechanism by which the outer mitochondrial membrane is permeabilized. The first model claims that non-specific pores at the inner mitochondrial membrane open to cause osmotic swelling of the mitochondrial matrix. This is followed by disruption of the outer membrane and the release of pro-apoptotic factors and cytochrome *c* (Petit, et al., 1998). Model number two says that the members of the Bcl-2 family regulate cytochrome *c* release. The anti-apoptotic proteins such as Bcl-2 and Bcl-x_L were found to prevent efflux of cytochrome *c* from the mitochondria by inhibition of Bax or Bak function or by binding of BH3-only proteins (Jurgensmeier, et al., 1998; Kluck, et al., 1997; Yang, et al., 1997). In contrast, Bax and Bak stimulated the opening of VDAC and allowed cytochrome *c* to pass (Shimizu, et al., 1999). Thus activation of Bax or Bak appears to be a predominant gateway to mitochondrial activation. The third model proposes a mitochondrial permeability transition-independent mechanism of cytochrome *c* release. The release may occur by modulation of the mitochondrial volume leading to swelling of this organelle. In

this scenario the mitochondria remain intact and maintain their membrane potential (Gogvadze and Orrenius, 2006). In the fourth model caspase-2 is the main player mediating cytochrome *c* release. Caspase-2 was shown to be associated with the mitochondria (Susin, et al., 1999) and to disrupt the inner mitochondrial membrane (Von Ahsen, et al., 2000). Interestingly, caspase-2 does not need its catalytic activity for this function (Robertson, et al., 2004). Although it was not attempted in this study to verify one or possible combinations of any of the models mentioned, cytochrome *c* efflux was investigated in respect to Bim_L and Bim_S. Immunostaining of cytochrome *c* in DU145-Bax cells revealed a leak of cytochrome *c* upon Bim expression (figure 14). Infection with $AdBim_L$, staining of cytochrome *c* and the mitochondria showed in the overlay that cytochrome *c* had been released from the mitochondria. In mock cells, which are Bax negative there was no sign for cytochrome *c* depletion upon Bim_L expression. In general, the same patterns were observed upon Bim_S expression. But there were more cells detected with cytochrome *c* depleted mitochondria. In the overlay, the strength of Bim_S became visible as it also induced cytochrome *c* release in some of the DU145 mock cells. This was confirmed by Western blot analysis, where cytochrome *c* could be detected in the cytosolic fraction upon expression of both Bim variants in DU145-Bax cells (figure 16). Release of cytochrome *c* occurred early, already 14h post infection with $AdBim_L$ cytochrome *c* could be detected in the cytosol. The cytosolic fraction of DU145-Bax cells was found to contain cytochrome *c* 10h post infection with $AdBim_S$. This rapid activation of the mitochondria shows an effective mode of action of Bim and might with regard to the time course of protein expression indicate that lower amounts of Bim_S as compared to Bim_L suffice to trigger the mitochondrial pathway.

The mitochondrial (intrinsic) pathway consists of release of cytochrome *c* and the breakdown of the mitochondrial membrane potential. Release of cytochrome *c* triggers the binding of dATP to Apaf-1 and the formation of the apoptosome, followed by the activation of caspase-9. Activation of caspase-9 was shown by Western blot analysis and flow cytometric measurements (figure 23, 25). In this study it could be observed that transfection of Bcl-2 and targeting of Bcl-2 to the mitochondria blocked the loss of the mitochondrial membrane potential (figure 22), suggesting that Bim induces the mitochondrial pathway. DNA fragmentation was also partially inhibited by Bcl-2 (figure 20). It can be concluded that the signals leading to mitochondrial activation are at least partially mediated upstream of the mitochondria and can be inhibited by anti-apoptotic proteins such as Bcl-2, e.g. targeted to the ER. This was found to be the case for release of cytochrome c but not for loss of the mitochondrial membrane potential.

It was shown by Western blot analysis that Bcl-2 blocked the Bim induced leak of cytochrome *c* from the mitochondria. When Bcl-2 was targeted to the mitochondria, only a

small amount of cytochrome *c* was released to the cytosol upon Bim expression (figure 25). A similar effect was achieved when Bcl-2 was present at the ER indicating that ER-mediated apoptosis may lead to secondary activation of the mitochondrial pathway. Both isoforms were blocked in their actions by Bcl-2 and this prevented the release of cytochrome c. Whether Bcl-2cb5 inhibited the effect of Bim by directly binding to this BH3-only protein or whether it blocked the multi-domain proteins Bak and Bax to become activated and therefore inhibited the pore formation at the mitochondrial membrane could not be clarified at this point. Of note, no protein interaction were studied here that might have elucidated the mode of Bim_L or Bim_S induced Bak/Bax activation.

There are data describing that cytochrome *c* release occurs before loss of the mitochondrial membrane integrity (Goldstein, et al., 2000). Others claim that cytochrome *c* can be released independently from breakdown of the mitochondrial membrane potential and activation of the caspases (Bossy-Wetzel, et al., 1998). Further it was reported that this process is conducted by the permeability transition pore and can be inhibited by overexpression of Bcl-2 (Susin, et al., 1996). It was also proposed that, without cytochrome *c*, apoptosis is attenuated and, consequently, the apoptosome is not obligatory for stress-induced apoptosis, but only acts as a caspase amplification system that is more important in certain cell types than others (Li, et al., 2000; Von Ahsen, et al., 2000).

In a next step, investigations concentrated on the question whether Bim-induced mitochondrial activation was dependent of Bax, Bak and inhibited by Bcl-2. In Bax negative mock transfectant cells, no cells were detected with mitochondrial permeability shift. Then again, in DU145-Bax cells, Bim_L induced breakdown of the mitochondrial potential, and Bim_S even twice as much. Approximately the same numbers were found in Bax-deficient DU145-Bak cells and their mock transfectants. Apart from the intensity, both isoforms were able to activate the mitochondrial pathway. In DU145-Bax re-expressing and DU145-Bak cells Bim induced breakdown of the mitochondrial membrane potential. In cells, which were either Bax negative or with low Bak expression, no cells could be determined with a mitochondrial shift, suggesting that Bim induces disruption of the mitochondria by activation of Bax or Bak. However, some reports say that Bax and Bak do not play a redundant role in activation of the mitochondria and cannot substitute for each other. BH3-only proteins were shown to induce apoptosis either via Bax or Bak. Nbk was found to kill cells in an only Bax-dependent manner (Gillissen, et al., 2003). Nbk is not the only BH3-only protein that is restricted to either Bax or Bak, also other members of this group depend on one of these two pro-apoptotic proteins to induce apoptosis. Therefore it was interesting to discover that Bim does not favour one of these two multi-domain proteins but is equally activating Bax and Bak to cause mitochondrial perturbation.

Studies with Bcl-2 targeted to the ER verified that it protects the mitochondria from a distance. There are established data suggesting that it can inhibit disruption of mitochondrial membrane potential (Annis, et al., 2001), release of cytochrome *c* from mitochondria (Hacki, et al., 2000), and oligomerization of Bax (Thomenius, et al., 2003). It has been demonstrated that Bax and ER targeted Bcl-2 do not interact during apoptosis, although ER-Bcl-2 inhibited apoptosis (Annis, et al., 2001). In another study, Bcl-2 at the ER was able to inhibit the oligomerization of a Bax mutant, which was constitutively localized on the mitochondria (Thomenius and Distelhorst, 2003). Since Bcl-2 localized at the ER and mitochondrial Bax are spatially separated, it is unlikely that they interact. However, the data obtained in this study together with the data generated by other groups showed that the mitochondrial cell death pathway induced by Bim could be prevented by Bcl-2. There is also evidence that BH3-only proteins may directly affect the mitochondria (Sugiyama, et al., 2002). These findings propose an alternative model in which Bcl-2 lies upstream of BH3-only proteins and impede them in activating Bax and inducing apoptosis. This model is also supported by recent reports that ER-targeted Bcl-2 prevents the mitochondrial localization of the BH3-only protein Bad (Thomenius and Distelhorst, 2003).

Expression of Bim in cells with mitochondria-localized Bcl-2 showed a decreased level of DNA-fragmentation (figure 20). The following investigation was supposed to answer the question whether the protection of the mitochondrial membrane potential by Bcl-2 was the reason for the reduced rate of apoptosis. 48h post infection with $AdBim_L$ high loss of the mitochondrial membrane potential was observed in the DU145 Bax neo cells. Bcl-2 targeted to the mitochondria reduced this effect by more than half, showing that Bcl-2 was able to protect the cells from Bim_L induced activation of the mitochondrial pathway. On the other hand, Bcl-2 localized to the ER could not save the mitochondria form losing their membrane potential. In these cells Bim_L induced a mitochondrial permeability shift almost as much as in cells without Bcl-2. Expression of Bim_S had a similar but stronger effect. Nearly all DU145-Bax neo cells infected with $AdBim_S$ were detected with mitochondrial permeability shift. Targeting Bcl-2 to the ER resulted in loss of the mitochondrial membrane potential for the majority of the cells. At first glance, this result raised doubts since there was no enhanced DNA fragmentation measured in these cells. But a possible solution came from other reports that some cells remain viable after permeabilization of the outer mitochondrial membrane (Holinger, et al., 1999). Therefore breakdown of mitochondria might not be necessary or sufficient for apoptosis (von Ahsen, et al., 2000). Only Bcl-2 at the mitochondria could protect the cells from losing their mitochondrial integrity upon Bim_S expression. It seems that the localization of Bcl-2 is important for its inhibitory effect, but the single fact of overexpressing Bcl-2 was not enough to fully neutralize Bim-mediated apoptosis induction at the mitochondria.

It was reported that Bcl-2 or Bcl-x_L can block mitochondrial membrane permeabilization induced by ectopically expressed BH3-only proteins or by Bax and/or Bak (Gross, et al., 1999). Therefore, it is possible that the apoptotic pathway is divided in two processes. Activation of pro-apoptotic proteins results in an initial release of approximately 10% of the cytochrome *c* from the intermembrane space followed by caspase activation. The second mechanism depends on the permeability transition leading to matrix swelling and consequent remodelling of the mitochondria, whereby all the cytochrome *c* is dumped (Bernardi and Azzone, 1981). These findings may underline the importance of the ER in the Bim pathway in the present study. Even though the mitochondria lost their membrane potential upon Bim expression (figure 22) in cells where Bcl-2 was targeted to the ER, these cells did not undergo apoptosis upon Bim expression (figure 20). It seems that the ER has to be accessible for Bim in order to induce apoptosis. If the ER is protected by Bcl-2, Bim can activate alternative pathways such as the mitochondrial cell death pathway as is indicated by the loss of the mitochondrial membrane potential, but this does not seem sufficient to lead to cell death. Further it might also support the model of the mitochondrial remodelling. This model implies that the permeabilization of the outer mitochondrial membrane does not lead to complete depletion of cytochrome *c* but requires the remodelling of the inner membrane. The structural reorganization of the mitochondria seems to be dependent of the permeability transition following activation of pro-apoptotic proteins to guarantee that cytochrome *c* release during apoptosis is quick and absolute. Studies have demonstrated that pro-apoptotic Bcl-2 family members remodel the mitochondrial structure (Scorrano, et al., 2002). The fact that Bcl-2 localized at the mitochondria did not completely inhibit apoptosis might support the theory that there are some alternative pathways and more importantly that the ER is available for Bim. Whereas Bcl-2 solidly prevented loss of the mitochondrial membrane potential, it did not inhibit apoptosis in the same strong way upon Bim expression. This could be another hint that the ER is an important station for Bim to fulfil its apoptotic function and that it does not rely exclusively on mitochondria as it is proposed for other BH3-only proteins.

Interestingly, transfected and targeted Bcl-2 could, at least partially, inhibit mitochondrial cell death but the processing of the caspases was merely blocked. Although this result was obtained by two different methods, the question needed to be answered how these caspases could be activated if cytochrome *c* release is prevented. It should be expected that caspase activation is completely blocked if there is no cytochrome *c* release. But it can be speculated that overexpressed Bcl-2 is able to inhibit cytochrome *c* release but nevertheless small amounts of cytochrome *c* could reach the cytosol, which is enough to activate the caspases to a certain extent. Small amounts of cytochrome *c* are not detectable in Western blots. And, as already mentioned the assumption that only a small

amount of the cytochrome *c* resides in the intermembrane space and most of it is found in the matrix (Bernardi and Azzone, 1981) might lead to the idea that permeabilization of the outer membrane and the liberation of few of these molecules is sufficient to activate the caspase cascade. For these reasons it is plausible that overexpression of Bcl-2 cannot fully inhibit cytochrome *c* release.

It should also be taken to account that in the case of Bim_S, inhibition of caspase-8 led to a partial protection of the mitochondria when Bcl-2 was overexpressed in the cell, regardless of its localization. Addition of the caspase-8 inhibitor reduced the number of cells with a mitochondrial permeability shift by half (figure 26). It was reported that caspase-8 can be recruited to the ER (Ng, et al., 1997) and can also be found at the outer mitochondrial membrane (Chandra, et al., 2004). Bcl-2 localized at the mitochondria diminished the amount of cells with lowered mitochondrial potential although Bim_S was present. Additional treatment with the caspase-8 inhibitor boosted this effect, only small amounts of cells were detected, which had lost the mitochondrial membrane potential. Based on these findings it could be speculated that Bim_S directly or indirectly activates caspase-8 to induce the breakdown of the mitochondrial membrane potential. Caspase-8 does not seem to be a target for Bim_L as inhibition of this caspase did not have an effect on mitochondrial activation (figure 26), but it can not be ruled out that caspase-8 is involved in its pathway through an amplification loop, e.g. through cleavage of Bid to tBid. Further aspects of caspase-8 and Bim will be discussed below.

5.3 Activation of caspases upon Bim expression

Various caspases are activated in different manner at different points of the apoptotic pathway, but nevertheless, activation of the caspases marks a crucial step in apoptosis. Mitochondrial membrane permeabilization and release of pro-apoptotic factors including cytochrome *c*, is involved in the activation of the caspases. However, there are debates about whether permeabilization of the outer mitochondrial membrane itself relies on or can occur independently of caspase activity (Breckenridge and Xue, 2004). Intermembrane space proteins are released at different time points following the apoptotic stimulus and some depend on caspases. This finding suggests that decisions are made upstream of mitochondria on whether and which pro-apoptotic factor is released form the intermembrane space (Breckenridge and Xue, 2004).

Emphasis of investigations in this thesis was put on caspase-3, caspase-9 and caspase-8. Caspase-9 is the key initiator caspase of the mitochondrial pathway and is recruited by APAF-1 into the apoptosome. Active caspase-9 cleaves thereby and activates caspase-3. Caspase-8 is essential for the death receptor pathway and mediates the mitochondrial

amplification loop. Caspase-3 represents the main downstream effector caspase and can be activated by either caspase-9 or caspase-8.

The activation of the three mentioned caspases was measured indirectly by detection of their cleavage products by use of Western blot analyses. The initiation of the caspase cascade was observed in DU145-Bax and DU145-Bak cells, presenting the capability of Bim to mediate cell death through both Bax and Bak (figure 16). DU145-Bax cells transfected with ER-targeted Bcl-2 showed resistance to caspase processing upon Bim expression (figure 23). These results were consistent with the measurements of DNA-fragmentation, where it was shown that these cells do not undergo apoptosis upon Bim stimulation in consequence of inhibition of the ER-mediated death pathway by Bcl-2cb5. Based on this finding it was concluded that the first station of Bim on this pathway is the ER, from where the death signal is transported to other compartments (figure 20). Further confirmation was achieved by flow cytometric measurements. Both, activation and inhibition of caspase-9, -3 and-8 were measured. To measure caspase activation, a FITC labelled caspase specific peptide substrate was used that binds to the activated enzyme. In all DU145 transfectants, an activation of the caspases could be measured (figure 23). The only exception was DU145-Bax Bcl-2cb5 cells, where none of the caspases were activated due to inhibition by Bcl-2cb5. The inverse picture was seen using caspase inhibitors individually for each of the three caspases. The apoptotic rate was dramatically reduced once the caspase inhibitor zVAD-fmk was added to the cells infected with $AdBim_L$ or $AdBim_S$ (figure 24). Lesser levels of inhibition were exerted by zDEVD-fmk and zIETD-fmk inhibition with zLEHD-fmk inhibition showing the least effect.

These inhibition studies of caspase-9 revealed that caspase-9 participates in Bim induced apoptosis in DU145-Bax and DU145-Bak cells (figure 15). Addition of zLEHD-fmk reduced apoptosis in both cell lines upon Bim_L expression by approximately one third. Upon Bim_S expression and treatment with caspase-9 inhibitor almost four times less apoptosis was measured in DU145-Bax as well as DU145-Bak cells. Yet, presence of Bcl-2 interfered with caspase-9 activation in principle, so that use of zLEHD-fmk did not show any major effect, no matter at which organelle Bcl-2 was localized. Measurement of caspase-9 activity upon Bim expression confirmed these data. Whereas cells without Bcl-2 showed elevated activation of caspase-9, Bcl-2 at the mitochondria diminished active caspase-9 by half. Bcl-2 targeted to the ER restrained the activation of caspase-9 even more. Compared to DU145-Bax neo, only one third of the cells were detected with activated caspase-9. These data suggest that both Bim variants seem to induce caspase-9 activation to the same extent. Caspase-3 is cleaved and activated by caspase-9 in the mitochondrial pathway. Therefore, this caspase should be a part of the Bim induced apoptotic pathway. Bax-dependent cell death was reduced in half of the cells when they

were, additionally to the adenovirus, treated with the caspase-3 inhibitor. Similar effects were found in DU145-Bak cells upon Bim expression and addition of zDEVD-fmk. Caspase-3 activity was also determined in context to Bcl-2 overexpression. Roughly, for both Bim isoforms cells overexpressing Bcl-2 resulted in a 50% reduction of the caspase-3 activity as compared to cells with no Bcl-2 overexpression. These results do not necessarily mean that caspase-3 has a bigger role than caspase-9 in respect to Bim. Higher caspase activation rates and stronger inhibition of apoptosis with zDEVD-fmk could be the result of an amplification loop. Caspase-8 connects the extrinsic and intrinsic pathway via Bid and can amplify the mitochondrial activation. Therefore, involvement of caspase-8 in the Bim pathway was also investigated. DU145-Bax cells treated with zIETD-fmk to inhibit caspase-8 presented upon expression of either of the Bim isoforms the same apoptotic rate as the ones treated with zDEVD-fmk. The same experiments in DU145-Bak revealed comparable apoptotic rates as detected in the DU145-Bax cells. Hence, it can be said that caspase-8 has a role in Bim induces apoptosis. So far in the work presented, both Bim splicing variants had a similar impact on cells except that Bim_S was more important in induction of cell death related events. Measurement of casapse-8 activation though, suddenly presented a difference between the two isoforms, which might have a differential impact on their pathway. Expression of Bim_L in DU145-Bax neo cells showed only a small quantity of cells with caspase-8 activity. Even less cells were detected with caspase-8 activity when Bcl-2 was present regardless of the localization. Expression of Bim_S in DU145-Bax neo cells demonstrated more than double as much cells with active caspase-8 as induced by Bim_L. But much less Bcl-2 transfectants with caspase-8 activation were observed when expressing Bim_S. These results gave the first clue that caspase-8 has a role in Bim_S induced apoptosis, which does not seem to be case for Bim_L. In consideration of reports that caspase-8 is located at the mitochondria and might have a great influence on mitochondrial activation, it was investigated whether inhibition of caspase-8 would have an effect on the loss of the mitochondrial integrity induced by Bim. Transduction of $AdBim_S$ in DU145-Bax neo cells led to 61% of cells with lowered mitochondrial membrane potential which did not change when zIETD-fmk was added to the cells. But overexpression of Bcl-2 and inhibition of caspase-8 prevented, however, the cells from loss of the mitochondrial membrane potential. In this constellation the caspase-8 inhibitor reduced the cells detected for mitochondrial permeability shift by half, no matter whether Bcl-2 was targeted to the mitochondria or the ER. Therefore, it may be assumed that Bcl-2 participates in the mechanism of caspase-8 activation initiated by Bim_S. Additional clues were given by the report that activation of caspase-8 might be achieved by a cytochrome *c* dependent way. It was proposed that caspase-6, an effector caspase like caspase-3, is the only caspase with the ability to activate caspase-8 after

cytochrome *c* release. In this case, caspase-8 would not need interaction with FADD and no formation of the DISC to be processed (Cowling and Downward, 2002). But as already discussed above no cytochrome *c* release could be detected by Western blot analysis in cells overexpressing Bcl-2, which makes this theory less likely, unless the minor part of cytochrome *c* kept in the intermembrane space would be enough to trigger this pathway. To make this point clear, further investigations especially about caspase-6 as the activator of caspase-8 would be necessary, but were not done in this work.

Detection of processed caspase-9, -3 and -8 by Western blot analysis revealed that these caspases are activated upon both Bim_L and Bim_S expression independently of the Bcl-2 expression level (figure 25). One possibility for the activation of these caspases despite of the presence of Bcl-2 could be a Bcl-2 insensitive feedback loop, where caspase-9 is activated, which in turn leads to activation of caspase-3. This effector caspase on the other hand is able to activate caspase-8. The other explanation could be taken from the proposition that only about 15% of the cytochrome *c* is located in the intermembrane space and the rest resides in the matrix (Bernardi and Azzone, 1981). Given that the inner mitochondrial membrane might not be permeabilized upon Bim expression, this small amount of cytochrome *c* might be able to induce caspase-9 activation and thereby trigger the activation of caspase-3 and -8. It should be noticed, that for the same time point (30h) there was more cleavage product detected for caspase-8 in cells transduced with $AdBim_L$ than for cells expressing Bim_S. It could be speculated that due to the strength of Bim_S and the fact that it is not post-translationally modified, active caspase-8 is already degraded at this time point.

All the results of the caspase investigations point to the conclusion that Bim requires mostly caspase-9 and -3 to execute apoptosis. Bim_S additionally seems to involve caspase-8 into these steps of cell death induction. Interestingly, blocking caspase-8 did not affect the activation of the mitochondrial and therefore the breakdown of the mitochondrial membrane potential by Bim_S. Bim_S induced permeabilization of the mitochondria could, however, be partially inhibited by usage of the caspase-8 inhibitor. DU145-Bax cells containing Bcl-2actA and also cells with Bcl-2cb5 showed a reduced number of cells with mitochondrial membrane potential loss. It seems that at this very early part of apoptosis induction Bim_S is able to activate an alternative, parallel apoptotic pathway where caspase-8 is activated upstream via the ER to trigger secondary activation of the mitochondrial pathway.

5.4 Bim_S causes ER stress and calcium release

The unfolded protein response is mediated through three ER transmembrane receptors, pancreatic ER kinase (PKR)-like ER kinase (PERK), activation transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). In healthy cells, all three ER stress receptors are maintained in an inactive state through binding to the ER chaperone BiP/GRP78. On accumulation of unfolded proteins, BiP dissociates from the three receptors, which leads to their activation and triggers the unfolded protein response. The unfolded protein response provides protection form cell death by reducing the accumulation of unfolded proteins and restores normal ER functioning (Schroder and Kaufman, 2005). However, when ER stress is excessive and prolonged cell death mechanisms are activated. Several mechanisms have been proposed for ER-induced cell death, including direct activation of proteases, kinases, transcriptions factors and Bcl-2 family proteins. Several investigations of ER stress induced apoptosis were performed in murine systems focusing on caspase-12, which is specifically cleaved upon induction of ER stress (Nakagawa, et al., 2000). There is no evidence of functional caspase-12 in human, although its mRNA was detected, but due to a frame shift mutation and disruption by a stop-codon and it is enzymatically inactive (Saleh, et al., 2004). Human caspase-4 was proposed to take the place of murine caspase-12 in humans in ER stress mediated cell death (Hitomi, et al., 2004). Caspase-4 was predominantly found at the ER membrane, but also at the mitochondria, which might imply additional ER-independent functions. Processing of caspase-4 was observed after exposure to ER stress inducing agents such as thapsigargin and tunicamycin in human neuroblastoma cells but not upon inducers of mitochondria-dependent dell death such as UV-irradiation and DNA damaging agents. However, caspase-4 knock down in HeLa cells had little effect on apoptosis induced by ER stress, implying that the importance of this caspase in ER stress is cell type dependent and so not sufficiently understood. In the investigations presented here, expression of Bim and inhibition of caspase-4 did not fundamentally block DNA-fragmentation (figure 24), questioning its suggested role, at least in Bim induced apoptosis. When Bcl-2 was not present, expression of Bim_L and treatment with caspase-4 inhibitor a minor reduction in cell death was observed. In the same settings with Bim_S a similarly decreased apoptotic rate was measured. In cells, where Bcl-2 was targeted to the mitochondria, the caspase-4 inhibitor to an only minor extent diminished apoptosis, when these cells were transduced with $AdBim_L$ or $AdBim_S$. These results reveal that caspase-4 is not the key caspase in ER stress-induced apoptosis, its inhibition helped to decrease cell death, but not in a major way. In the presence of Bcl-2 the effect of the caspase-4 inhibitor was even smaller, probably because overexpressed Bcl-2 anyhow partially inhibited apoptosis. When Bcl-2 was targeted to the ER, no effect of the caspase-

4 inhibitor could be detected, neither upon Bim_L nor Bim_S expression. This was not surprising since these cells did not show relevant apoptosis induction or show by analyses of DNA-fragmentation upon Bim expression. Further studies are needed to clarify a potential role of caspase-4 in Bim induced ER stress. The influence of capase-4 inhibition on mitochondrial membrane potential loss and detection of caspase-4 cleavage products could give some insight on participation of caspase-4. A potential candidate for the role as the mediator of ER stress induced apoptosis is caspase-8. It is thought to act as initiator caspase at the ER and to contribute to mitochondria-ER crosstalk. It was found to localize to the outer mitochondrial membrane in an active state. Caspase-8 localized at mitochondria may activate caspase-3, which then processes caspase-9 and thus might contribute to the amplification loop. As another substrate of casapse-8, Bap31 (Bcl-2 associated protein 31) was identified. It was observed in a regulatory complex at the ER with Bcl-2 and caspase-8 (Ng, et al., 1997). Cleavage of this integral ER protein generates a p20 fragment that has pro-apoptotic features. The p20 fragment, which remains in the ER membrane, may mediate mitochondrion-ER crosstalk through a Ca^{2+}-dependent mechanism (Chandra, et al., 2004). It causes the release of Ca^{2+} from the ER, which is in turn taken up by mitochondria and triggers the release of cytochrome *c* and caspase activation (Rao, et al., 2004). Overexpression of Bax or Bak led to Ca^{2+} efflux from the ER, Ca^{2+} influx into the mitochondria resulting in cell death, that can be prevented by Bcl-2 (Nutt, et al., 2002). The expression of Bcl-2 decreased the amount of Ca^{2+} that could be released from intracellular stores, regardless of the mode of store depletion, the cell type or the species from which Bcl-2 was derived. The response to ER stress agent thapsigargin revealed that Bcl-2 increased the permeability of the ER membrane. Bcl-2 is said to inhibit apoptotic mechanisms downstream of cytochrome *c*, probably at the level of the ER (Foyouzi-Youssefi, et al., 2000). The ER-mitochondria crosstalk might be used for mitochondrial amplification of ER initiated apoptosis. Considering the findings described, the following pathway might apply for Bim_S: Active caspase-8 in the outer mitochondrial membrane cleaves and activates Bap31 to p20, upon which Ca^{2+} is released from the ER. Liberated Ca^{2+} is absorbed by the mitochondria causing their permeabilization, which might lead to apoptosis. Several studies have demonstrated that members of the Bcl-2 family also integrate into the ER membranes, where they regulate the transfer of ER Ca^{2+} to mitochondria and ER stress signals (Oakes, et al., 2006). Whereas anti-apoptotic members of the Bcl-2 family can prevent alteration of Ca^{2+} homeostasis, pro-apoptotic members promote Ca^{2+} mobilization from the ER to mitochondria during apoptosis, perhaps by regulating of the activity of the ER inositol trisphosphate receptor. This might be important for the mitochondrial permeability transition pore opening and intermembrane space protein release (Breckenridge and Xue, 2004; Mathai, et al., 2005;

White, et al., 2005).

Considering the data obtained in this thesis, the just mentioned process does not seem to be main pathway, but lead to an amplification and acceleration of the death signal. For further investigations about the role of Bim in ER stress, Bim should be expressed in the presence of the caspase-8 inhibitor and subsequent detection for Bap31 should not show the p20 fragment. This would be a further clue that at least Bim_S mediates apoptosis by inducing ER stress. This hypothesis is supported by the finding that upon Bim_S expression DU145-Bax neo and DU145-Bax Bcl-2actA cells showed upregulation for Bap31, but not in the cells, that express Bcl-2 at the ER (figure 28). This confirms that Bcl-2 is able to inhibit induction of ER stress when it is localized at the ER. Further, this is in line with the finding that DU145-Bax neo and DU145-Bax Bcl-2actA cells displayed Ca^{2+} release upon Bim_S expression (figure 27). Surprisingly, Western blot analysis of these cells transduced with $AdBim_L$ presented under on conditions the same picture as described for Bim_S. It might therefore be speculated that Bim_L does also target the ER, leading to upregulation of Bap31, but fails to induce ER stress and thus also calcium release. The slight induction of Bap31, which was detected under off conditions might come from the transduction of the cells with the adenovirus. Supplementary clues that this hypothesis of Bim_S but not Bim_L acting through the ER pathway were given by data showing that capase-8 is activated upon Bim_S expression but not in cells infected with $AdBim_L$ (figure 23). Moreover, perturbation of the mitochondria could be diminished by capase-8 inhibition when Bim_S was expressed in the cells, which was not the case upon Bim_L expression (figure 26). Overexpression of the p20 Bap31 fragment triggered Ca^{2+} release and sensitized mitochondria for caspase-8 induced apoptosis (Breckenridge, et al., 2003). Activation of the ER and the mitochondria might nevertheless be partially caspase-8 independent, although the mechanisms described for Bim so far, relies on caspase activation. Caspase-8, Bap31 and Bcl-2 or Bcl-x_L that were found to form a complex (Ng, et al., 1997). Formation of this complex can be blocked by the BH3-only protein Spike (Mund, et al., 2003).

Another ER stress-induced cell death modulator is CHOP, a transcription factor induced during ER stress (Kaufman, 1999; Wang, et al., 1996). Lack of CHOP provides partial resistance to ER stress-induced apoptosis (Zinszner, et al., 1998). CHOP promotes ER stress mediated apoptosis by repressing Bcl-2 gene expression, which increases the proportion of pro-apoptotic proteins in the cell and facilitates their activation. How CHOP induces apoptosis though, is unclear. While capable of inducing apoptosis and contributing to cell death in several scenarios involving ER stress, CHOP is not essential for cell death induced by ER stress (Harding, et al., 2003). Overexpression of CHOP has been shown to induce apoptosis, which was linked to the activation and mitochondrial

translocation of Bax. Vice versa, overexpression of Bcl-2 could block CHOP induced apoptosis (McCullough, et al., 2001). This could not be confirmed by overexpression of Bim in DU145 cells. Under on conditions, both Bim_L and Bim_S induced upregulation of CHOP independently of the Bcl-2 status. A further ER protein involved in ER stress is the chaperone protein GRP78/BiP (immunoglobin heavy chain binding protein). It maintains ER function, facilitates protein folding and protects cells from toxic insults, hence has anti-apoptotic abilities. BiP is localized inside the ER lumen, but it is also found on the cell surface, especially in prostate cancer cells (Mintz, et al., 2003). BiP functions as a main regulator of the unfolded protein response by binding to and preventing the activation of all three stress sensors IRE1, PERK, ATF6 (Kaufman, 1999). Upon ER stress these sensors are released form BiP and become activated. Additionally, BiP inhibits apoptotic signals at least in part by blocking caspase activation (Rao, et al., 2002). All in all, BiP has protective function against cytotoxic insults, it can protect cells against apoptosis caused by disturbance of ER homeostasis. Detection of BiP by Western blot analysis demonstrated increased levels of this protein upon expression of both Bim isoforms. No differences were found in-between cells with or without Bcl-2 overexpression. The strong expression of BiP might indicate that this protein is induced and regulates ER stress induced by Bim. BiP is also believed to regulate ER Ca^{2+} storage (Lievremont, et al., 1997). Measurement of elevated cytosolic calcium revealed that the short form of Bim induced calcium release form the ER in a time dependent manner. DU145-Bax neo transduced with $AdBim_S$ showed increased calcium levels making regulation of calcium fluxes by BAP31 in this setting unlikely. Within 8h the cytosolic Ca^{2+} levels increased considerably upon Bim_S expression in these cells. Bcl-2 localized at the mitochondria partially interfered with Bim_S induced calcium leak. Noticeable, but still less calcium fluxes were measured in DU145-Bax Bcl-2actA cells increasing slightly in the same time frame. It was therefore not surprising that DU145-Bax Bcl-2cb5 cells, which express Bcl-2 at the ER were resistant to Bim_S induced calcium release Consequently, when the ER was blocked by Bcl-2, Bim_S was not able to promote leakage of calcium from this organelle. Expression of Bim_L did not induce calcium release in none of the three cell transfectants at any time point. Taking in account that BiP can block ER signalling proteins, Ca^{2+} release and caspase activation, the long variant of Bim might not be able to counteract. In various tumour cells, among them prostate cancer cell line DU145, which were used in the present studies, highly augmented BiP expression was determinated (Arap, et al., 2004; Misra, et al., 2005). The ability of Bim_S to induce stronger caspase-8 activation as compared to Bim_L might lead to calcium release from the ER. The second reason might be the strength of Bim_S to induce apoptosis in comparison to Bim_L, which was constantly evident throughout the investigations. Therefore, Bim_S might be able to overcome a

putative protective effect of BiP.

Bcl-2 family members including Bcl-2 Bcl-x_L, Bax, Bak and Nbk have been shown to associate with the ER, suggesting that Bcl-2 family proteins operate at the ER to regulate calcium homeostasis and apoptotic cell death (Breckenridge, et al., 2002; Ng, et al., 1997).

5.5 Concluding remarks

Although further investigations must be conducted to elucidate the molecular basis and interactions of Bim_L and Bim_S, the following scenario is supported by the data provided in this study: A cell death signal activates Bim_L resulting in its release from the microtubule and translocation to the ER. There it activates an ER-associated apoptosis signal that results in activation of Bak and Bax. It is unclear whether this occurs at the mitochondria or the ER. At the mitochondrion Bak and Bax mediate loss of the mitochondrial membrane potential and the release of cytochrome *c*. The liberation of cytochrome *c* activates the apoptosome and triggers the activation of caspase-9, which mediates the cleavage of caspase-3. It could not be clarified whether ER and mitochondrial events happen sequentially or represent two separate pathways. Although is was recently shown by cell survival assays that Bim is the most critical initiator of ER stress induced apoptosis (Puthalakath, et al., 2007), the mechanism of ER/mitochondria crosstalk is nevertheless enigmatic.

Bim_L can be antagonized by Bcl-2, although this anti-apoptotic protein cannot entirely block the induction of apoptosis. The prevention of cell death by Bcl-2 seems to depend on its localisation. Expressed at the mitochondria, Bcl-2 could only partially inhibit apoptosis. While cytochrome *c* release from mitochondria was inhibited by both Bcl-2actA and Bcl-2cb5, only Bcl-2actA interfered with loss of the mitochondrial membrane potential. The reason for this might be that Bim_L still manages to induce mitochondrial activation to a certain extent, which is reflected in the reduced but yet detectable DNA-fragmentation. When Bcl-2 resides at the ER, Bim_L was not able to conduct apoptosis, suggesting that ER-stress induced apoptosis is parallely activated to the mitochondrial apoptosis pathway.

Bim_S seems to mainly use the same pathway as Bim_L by activation of the mitochondrial cell death pathway. Just as for Bim_L, Bcl-2 expression at the mitochondria can prevent activation of the mitochondrial apoptosis pathway and therefore apoptosis. But there is one dramatic difference between the pathway of Bim_L and Bim_S. According to the findings made in this project, caspase-8 is the chief helper of Bim_S. Upon activation, Bim_S not only induces the activation of the multi-domain proteins Bak and Bax but additionally also of caspase-8, whether this might be in a direct or indirect way. Only upon Bim_S expression

IETD-fmk could inhibit loss of the mitochondrial membrane potential. Moreover, Bim_S induced a higher percentage of cells with caspase-8 activation as shown by binding of fluorescing IETD peptide substrate. Localization of Bcl-2 at the ER fully blocked Bim_S mediated apoptosis. Activation of the ER apoptosis signal seems to be triggered simultaneously to the activation of the mitochondrial apoptotic events. Bcl-2 seems to grant survival at this position even against such a strong killer as Bim_S. By targeting the ER, Bim_S causes the depletion of the calcium stores causing ER stress as an additional apoptotic pathway to its activation of the mitochondrial cell death pathway. Bim_L does activate caspase-8 only in a small number of cells although it induces upregulation of the ER stress proteins such as BiP, CHOP and Bap31. ER stress is said to be followed by calcium release and subsequent uptake by the uniporters of the mitochondria. Uptake of calcium into the mitochondria causes swelling of the matrix and can lead to disruption of the membranes. Active capase-8 was found by others to be integrated into the outer mitochondrial membrane, where it assists on permeabilization of the mitochondria (Chandra, et al., 2004). Additionally, it is also possible that caspase-8 activates Bid by cleavage to tBid, which can not only trigger the activation of the multi-domain pro-apoptotic proteins Bak and Bax but can also form pores at the outer mitochondrial membrane enhancing the disruption of these organelles. Therefore, Bim_S might have three possibilities or any combinations of those to induce and most importantly to ensure cell death once it is activated. Based on the results obtained in the present study, the following model is proposed:

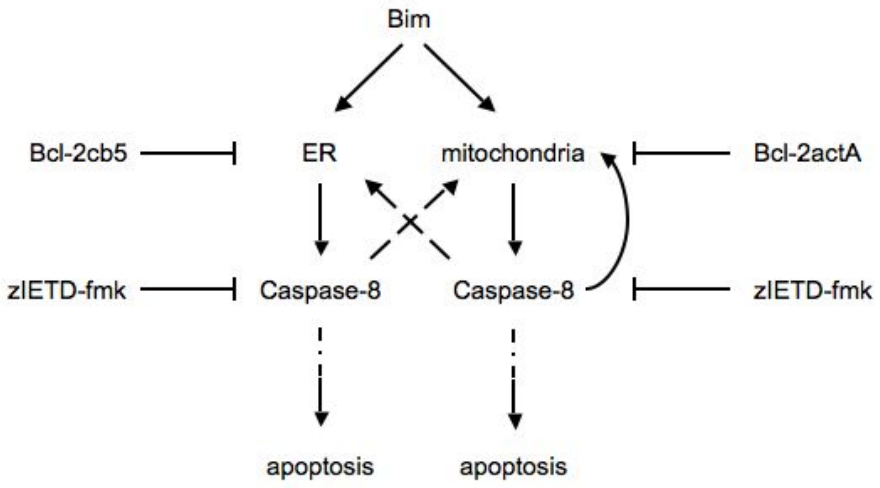

Figure 29: Model for Bim induced apoptosis

Although some steps of Bim induced apoptotic pathway were illuminated, there are still a lot of questions to be answered. For example, if other anti-apoptotic proteins such as Bcl-

x_L are also able to inhibit the pathway of Bim or how Bim causes ER stress. Further it would be interesting to know, by which mechanism Bim_S induces the release of Ca^{2+} from the ER and the activation of caspase-8. The ER might mark the point where Bim_L and Bim_S follow different pathways. Bim_L might activate Bax and Bak already at the ER and not later at the mitochondria which then leads to the permeabilization of the mitochondria followed by the release of cytochrome *c* and finally to apoptosis. Bim_S does not only activate Bax and Bak just like Bim_L, but also induces a leak of calcium from the ER, which is taken up by the mitochondria and enhances the death signal. It can be speculated if this additional step of calcium mobilisation might be the reason for the potency of Bim_S.

One reason for the strength of Bim_S might lay in the fact that it is not bound and therefore not regulated by the dynein motor complex of the microtubule. An incoming death signal might activate Bim_S directly, whereas Bim_L would have to be released from the microtubule. Another explanation could be taken from the report that Bim_S is subjected to any post-translational modification, but is directly active after expression as opposed to Bim_L and Bim_{EL} (Weber, et al., 2007). Finally, the structure of Bim_S could be responsible for its strong killing ability. It was speculated that the intensity by which a BH3-only protein induced the apoptotic pathway is depending on its affinity to anti-apoptotic proteins. No such data are, however, at presence available comparing Bim_S and Bim_L. Binding assays revealed that Bim and Puma, which are both strong killers, had high affinity to all anti-apoptotic family members and could neutralize all the anti-apoptotic proteins, whereas the less potent BH3-only proteins had a more restricted binding spectrum (Chen, et al., 2005). On the other hand, Noxa only bound to Mcl-1 and Bfl-1/A1 and turned out to be a weak killer, Nbk neutralized Bcl-w, Bcl-x_L, but not Bcl-2 or Mcl-1. Therefore it was suggested that limited targeting of anti-apoptotic proteins correlates with the apoptotic strength of the BH3-only proteins (Chen, et al., 2005). Such a mechanism might therefore also explain the higher potency of Bim_S to induce apoptosis as compared to Bim_L. The exact interplay with the anti-apoptotic proteins needs further extensive investigations and cannot be explained at this point. Further, it can be speculated that higher induction of apoptosis by Bim_S might be due to the additional amplification of the death signal by the release of calcium from the ER. As presented in this study, Bim_S did not only upregulate ER stress proteins but also triggered elevated cytosolic calcium levels. Calcium released by the ER is known to be absorbed by the mitochondria (Szalai, et al., 1999), which in turn contributes to mitochondrial permeabilization and the release of cytochrome *c* and other apoptotic factors.

The data presented in this thesis can promote Bim as a physiologically relevant target in tumour therapy.

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7 Appendix

7.1 Posters and presentations

"Oncodeath" meeting

Gaby Forro, Bernd Gillissen, Antje Richter, Bernd Dörken and Peter Daniel

The role of the BH3-only protein Bim in the intrinsic apoptotic pathway, oral presentation Innsbruck, Austria, 2006

European students' conference

Gaby Forro, Bernd Gillissen, Antje Richter, Bernd Dörken and Peter Daniel

The effects of Bim in apoptosis, oral presentation, Berlin, Germany, 2006

14th euroconference on apoptosis

Gaby Forro, Bernd Gillissen, Antje Richter, Bernd Dörken and Peter Daniel

Effects of Bim in the apoptotic pathway, Sardinia, Italy, 2006, poster presentation

European Workshop on Cell Death

Gaby Forro, Bernd Gillissen, Antje Richter, Bernd Dörken and Peter Daniel

The role of the BH3-only protein Bim in apoptosis, Kerkrade, The Netherlands, 2006, poster presentation

13th euroconference on apoptosis

Gaby Forro, Bernd Gillissen, Antje Richter, Bernd Dörken and Peter Daniel

The role of the BH3-only protein Bim in the intrinsic apoptotic pathway, Budapest 2005, poster presentation

European students' conference

Gaby Forro, Bernd Gillissen, Antje Richter, Bernd Dörken and Peter Daniel

The effects of Bim in apoptosis, Berlin, Germany, 2005, poster presentation, winner of the session prize

"Oncodeath" meeting

Gaby Forro, Bernd Gillissen, Antje Richter, Bernd Dörken and Peter Daniel

Induction of apoptosis by the adenoviral overexpression of the BH3-only protein Bim, Montpellier, France, 2005, oral presentation

7.2 Publications

The cytoplasmic domain of teneurin-2 has a nuclear function and represses zic-1 mediated transcription

Claudia Bagutti; **Gaby Forro**; Jacqueline Ferralli; Beatrix Rubin and Ruth Chiquet-Ehrismann

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